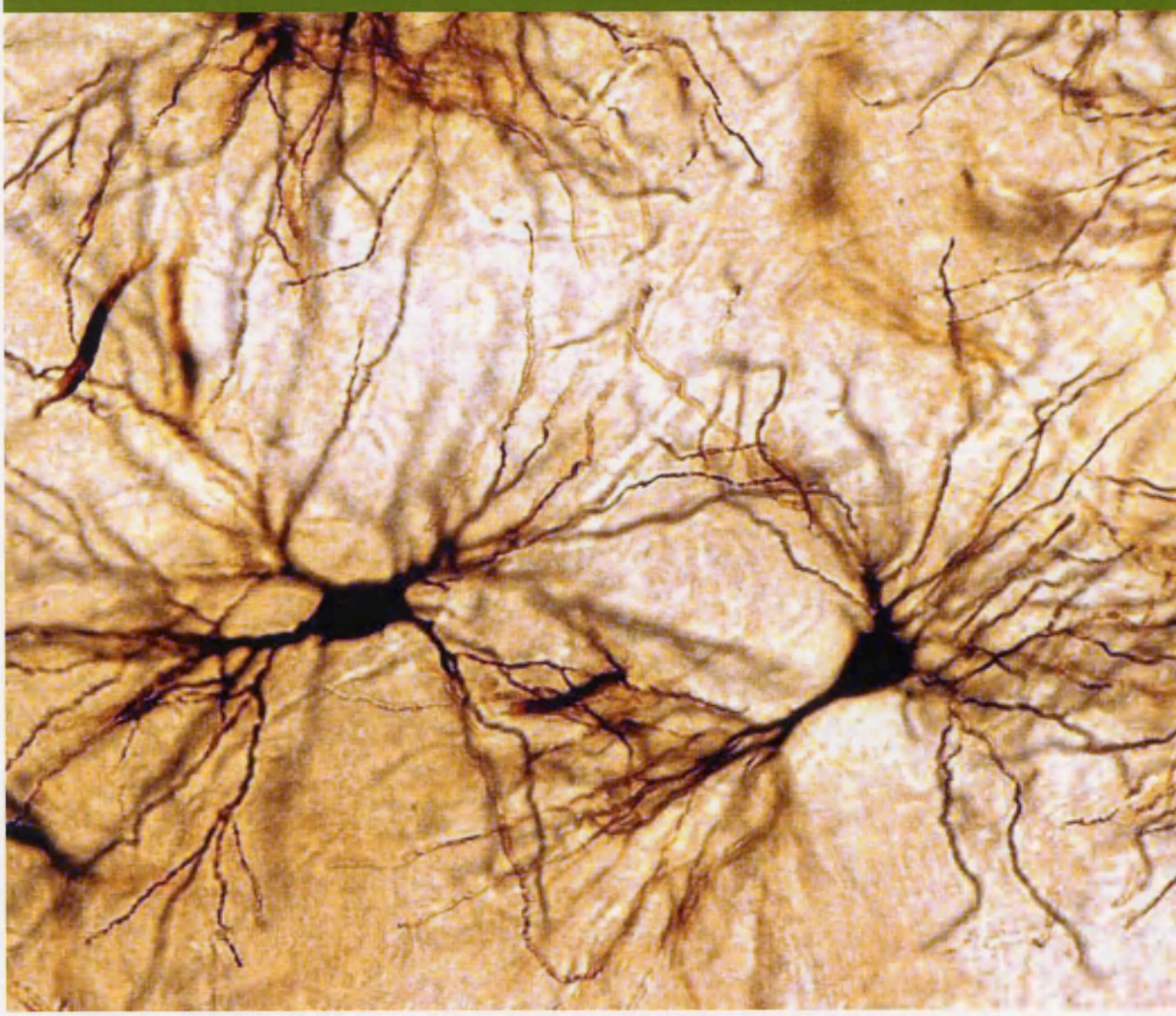
2016 / Volume 54 / Number 3
ISSN 1641-4640

Folia NEUROPATHOLOGICA

www.folianeuro.termedia.pl

Official Journal of Mossakowski Medical Research Centre Polish Academy of Sciences
and
Polish Association of Neuropathologists



ISSN 1641-4640

Folia Neuropathologica

TERMEDIA Publishing House

Folia Neuropathologica
former *Neuropatologia Polska*

Official Journal of Mossakowski Medical Research Centre Polish Academy of Sciences and Polish Association of Neuropathologists

The journal is partly financially supported by the Ministry of Science and Higher Education

termedia

Termedia Publishing House
Poznań office
Kleeberga 2, 61-615 Poznań, Poland
phone/fax: +48 61 822 77 81
e-mail: termedia@termedia.pl
www.termedia.pl
www.folianeuro.termedia.pl

Warsaw office
Warsaw Financial Center
Floor 11, Room 1149
Emilii Plater 53, 00-113 Warszawa, Poland
phone/fax: +48 22 827 75 14
e-mail: biuro.warszawa@termedia.pl

President of the Management Board of the Termedia Publishing House
Janusz Michalak

Scientific Director of the Termedia Publishing House
Maciej Banach

Production Editor
Marzena Demska
e-mail: m.demska@termedia.pl

Marketing and Advertising Department
Renata Dolata
phone: +48 61 822 77 81 ext. 508
e-mail: r.dolata@termedia.pl

Distribution Subscription Department
Jolanta Jankowiak
phone: +48 61 656 22 00
e-mail: prenumerata@termedia.pl

Impact Factor for Folia Neuropathologica equals 1.233
MNiSW score for Folia Neuropathologica equals 20
Index Copernicus Value 2014 for Folia Neuropathologica equals 22.61 (154.82)
Position in Index Copernicus ranking systems available at http://www.indexcopernicus.pl

Abstracted and indexed in Index Medicus/MEDLINE, Neuroscience Citation Index, SciSearch, Research Alert, Chemical Abstracts, EMBASE/Excerpta Medica, Polish Medical Bibiography, Index Copernicus

Print run: 450 copies

Folia
Neuropathologica
2016; 54/3: 197-350

Contents

Review paper

Folia Neuropathologica

Heterogeneity of histopathological presentation of pilocytic astrocytoma – diagnostic pitfalls. A review

Ewa Matyja[1], Wiesława Grajkowska[2], Katarzyna Stępień[1], Ewa Naganska[1]

[1]Department of Experimental and Clinical Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences,
[2]Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland

Folia Neuropathol 2016; 54 (3): 197-211 DOI: 10.5114/fn.2016.62530

Abstract

Pilocytic astrocytomas (PAs) are the most frequent primary astroglial tumours affecting children and adolescents. They occur sporadically or in association with a genetically determined syndrome – neurofibromatosis type 1. Classic PA usually manifests as a well-circumscribed, often cystic, slowly growing tumour, which corresponds to WHO grade I. The majority of pilocytic tumours arise along the neuraxis, predominantly in the cerebellum. They are associated with favourable long-term outcome or spontaneous regression, even after incomplete resection. However, the behaviour and prognosis might also be related to tumour histology and location. Pilomyxoid astrocytoma (PMA) represents a variant of classical PA with more invasive growth and increased risk of recurrences and dissemination.
Typically, PAs exhibit distinct histology with biphasic architecture of loose, microcystic and compact, fibrillary areas. However, some tumours arise in an uncommon location and display heterogeneous histopathological appearance. The morphological pattern of PA can mimic some other glial neoplasms, including oligodendroglioma, pleomorphic xanthoastrocytoma, ependymoma or diffuse astrocytoma. Not infrequently, the advanced degenerative changes, including vascular fibrosis, and recent and old haemorrhages, may mimic vascular pathology. Sometimes, the neoplastic piloid tissue can resemble reactive gliosis, related to long-standing non-neoplastic lesions. Not infrequently, PA exhibits histological features typical for anaplasia, including necrosis, mitoses and glomeruloid vascular proliferation that can suggest a diffuse high-grade glioma. However, even those PAs that lack distinct histological features of anaplasia can behave unpredictably, in a more aggressive manner, with leptomeningeal spreading.
Genetic alterations resulting in aberrant signalling of the mitogen-activated protein kinase (MAPK) pathway have been considered to underlie the development of PAs. The most commonly identified KIAA1549-BRAF fusion is important for appropriate tumour molecular diagnosis.
In this paper we summarize the clinicopathological presentation of PAs, with emphasis on their heterogeneous morphology, based on our own experience in the field of surgical neuropathology and the literature data. Diagnosis of pilocytic tumours requires careful analysis of clinical, histopathological and molecular features to avoid misinterpretation of these benign neoplastic lesions.

Key words: *pilocytic astrocytoma, pilomyxoid variant, low-grade glioma, heterogeneous morphology.*

Communicating author
Ewa Matyja, MD, PhD, Department of Experimental and Clinical Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, 5 Pawinskiego St., 02-106 Warsaw, Poland, phone/fax: +48 22 668 55 32, e-mail: ematyja@imdik.pan.pl

Introduction

Pilocytic astrocytoma (PA) is a well-circumscribed, well-differentiated, slowly growing tumour, corresponding to WHO grade I. It is included in the group of "other astrocytic tumours" in the revised 4th edition of the current 2016 WHO Classification of Tumours of the Central Nervous System [25]. Typically, PAs exhibit a non-diffuse pattern of cell growth, although they may locally infiltrate leptomeninges or adjacent brain tissue.

The vast majority of cases are associated with benign clinical behaviour and a favourable long-term outcome, particularly those of cerebellar location and complete surgical resection [27-29,60]. However, some tumours, despite their benign histology, may behave more aggressively. The pilomyxoid variant of pilocytic astrocytoma (PMA) is suggested to represent a subtype with more invasive growth and increased risk of recurrences [49,50]. Occasionally, the classic PA may exhibit the distinct histological features of anaplasia such as necrosis, mitoses and vascular proliferation [10,25,31,53,82,90,101]. Some other factors, including partial resection, brainstem location and adult age, are also associated with a worse prognosis [27].

Generally, PA is characterized by distinct clinicopathological features, but not infrequently it manifests heterogeneous appearance, including older age of patients, uncommon location, lack of classical biphasic pattern, presence of nuclear atypia, mitoses, necrosis and vascular glomeruloid changes. In such cases the accurate diagnosis may be challenging [60]. Moreover, the biological behaviour of pilocytic astrocytomas, independently of their clinical course and morphology, is not always predictable.

This report evaluates our own experience in the field of surgical neuropathology of PA and reviews the literature. The correct diagnosis of PA has important therapeutic and prognostic implications; thus the careful analysis of clinical, histopathological and molecular features is required to avoid misinterpretation in differential diagnosis.

Incidence

Pilocytic astrocytomas occur most commonly in paediatric and young adults in the age group 0-19 years [71], constituting 10% of cerebral and 85% of cerebellar tumours. They are often associated with neurofibromatosis type 1 (NF1), especially those cases developing in the optic/chiasmatic region. The latter account for at least 40% of cases associated with NF1 [58]. Pilocytic astrocytomas are rarely encountered in patients older than 50 years [20,37,59]. The oldest patients with pathologically confirmed PA were an 86-year-old woman [63] and an 85-year-old man with post mortem diagnosis of PA in the brainstem [20].

Localization

Pilocytic astrocytomas may arise along the entire neuraxis. They affect preferentially the cerebellum but may arise in the optic pathway, hypothalamus, brainstem and spinal cord [27,60]. Less commonly they are located in the basal ganglia and cerebral hemispheres. In the paediatric age group, PAs develop preferentially in hemispheres of the cerebellum, followed by the brain stem and spinal cord. Occasionally, PA may develop in the neurohypophysis/suprasellar region [79] and pineal gland [8]. Brain stem tumours usually appear as a compact dorsal exophytic mass lesion [5]. Exceptionally, paediatric PA may occur as a cerebellopontine angle mass, well separated from the brain stem [65,92].

In adult patients, the majority of PAs are located supratentorially [14,18], less commonly in the cerebellum [96,100], cervicomedullary region [103] or other parts of the spinal cord [37,74,86]. Occasionally, PA in adult patients are associated with haemorrhages and manifest as spontaneous intracranial haemorrhage [57,59].

Clinical and radiological findings

The clinical presentation of PA depends on tumour location. The neuroimaging findings of PAs, including conventional and advanced magnetic resonance imaging (MRI) sequences, depend on the tumour size, location and tendency to infiltrate the surrounding structures [22]. Typically, PA occurs on computed tomography (CT) and MRI as a well-demarcated, contrast-enhancing lesion that reveals characteristic solid and micro- or macrocystic components [98]. The cyst with an enhancing mural nodule is a classic radiological feature. The solid part usually shows homogeneous contrast enhancement. On MRI, the tumour appears as hypo- or isointense lesion on T1-weighted images and hyperintense on T2-weighted images [55]. Tumour calcifications occur only occasionally.

In some PA cases, the neuroradiological images may be unusual, including small cyst formation, heterogeneously or irregularly enhancing tumour nodules and internal haemorrhages [68]. Uncommonly, the radiological features of PAs can resemble high-grade gliomas.

Histopathology

The borders of PAs are usually well-defined, but sometimes the neoplastic infiltration of adjacent brain tissue can be seen (Fig. 1A). Tumours of cortical location may demonstrate invasion of the leptomeninges with extensive desmoplastic reaction (Fig. 1B).

Typically, PA exhibits a well-recognizable biphasic pattern with a mixture of loose microcystic and more compact regions (Fig. 1C, D). Such distinct biphasic architecture is particularly evident in tumours of cerebellar location. Numerous small cysts of different size and shape, filled with eosinophilic, myxoid material, could be seen (Fig. 1E, F). The microcystic, loose-textured areas contain stellate astroglial cells resembling protoplasmic astrocytes and globular, mulberry-shaped, eosinophilic granular bodies (EGBs) or hyaline globules (Fig. 2A). Eosinophilic granular bodies are formed by aggregates of tiny granular bodies (Fig. 2B) situated within the astrocytic processes. They exhibit periodic acid-Schiff, α_1-antichymotrypsin and α_1-antitrypsin reactivity. The compact parts of the tumour are mostly composed of bipolar piloid cells with delicate "hair-like" processes. Some more solid areas may contain a large amount of eosinophilic structures corresponding to EGBs and/or hyaline droplets (Fig. 2C) or Rosenthal fibres (Fig. 2D). The latter appear as eosinophilic, hyaline, corkscrew-like structures (Fig. 2E), which accumulate within the tumour cell processes. Ultrastructurally, they are electron-dense granular masses surrounded by intermediate glial filaments. Rosenthal fibres are composed of α-B-crystallin and do not express glial fibrillary acidic protein (GFAP) positivity or exhibit only peripheral immunostaining. Some regions are composed entirely of a large number of Rosenthal fibres. It is well known that both EGB and Rosenthal fibres are not specific for PA; nevertheless, they can serve as a suitable marker of slow-growing lesions. Eosinophilic granular bodies are often present in other non-diffuse glial and glio-neuronal tumours, including ganglioglioma and pleomorphic xanthoastrocytoma. Rosenthal fibres are also a frequent component of chronic reactive piloid gliosis associated with other neoplastic brain lesions, i.e. craniopharyngioma, cerebellar haemangioblastoma or spinal ependymoma. Similar looking gliosis might also be encountered in the pineal cyst and various non-neoplastic lesions associated with the reactive response of astroglia. It is noteworthy that both EGBs and Rosenthal fibres are not required for the diagnosis of PA.

Immunohistochemically, the neoplastic cells of PAs show strong GFAP immunoreactivity (Fig. 2F), S-100 protein and OLIG2 positivity.

The classic biphasic architecture was evident in the majority of PAs cases. However, some tumours are composed predominantly or even entirely of one growth pattern. They display either microcystic or solid appearance, with or without EGBs or Rosenthal fibres. Such monomorphic morphology can be confusing and challenging for neuropathologists.

Not infrequently, PAs contain areas of oligodendroglioma-like pattern (Fig. 3A) with a honeycomb picture formed by uniform cells with a clear perinuclear halo (Fig. 3B). Such morphology mimicking oligodendroglioma predominates in the histological picture of some pilocytic astrocytomas [95].

Pilocytic astrocytomas usually show signs of degenerative changes, typical for slowly growing, long-standing lesions. They include not only presence of Rosenthal fibres and EGBs, but also focal increase of cellularity with nuclear hyperchromasia (Fig. 3C) and cellular pleomorphism (Fig. 3D). Large, pleomorphic, often multinucleated cells reflect advanced degenerative changes which have no prognostic significance (Fig. 3E). Some cells, corresponding to the cells known as "pennies-on-a-plate", exhibit multiple nuclei arranged circumferentially in the cytoplasm (Fig. 3F). Occasionally, thin or psammomatous calcifications can be found (Fig. 3G, H). It is a frequent finding in optic nerve, hypothalamic/thalamic and superficially located cerebral tumours. Densely calcified PAS of the brainstem [15] and unique cases of intraventricular PA with densely calcified tissue and psammomatous bodies were reported [47,52].

Some tumours exhibit a focal angiocentric pattern with ependymoma-like perivascular arrangement of neoplastic cells (Fig. 4A). Such a pattern might be seen in oligodendroglioma-like areas (Fig. 4B). Occasionally, the neoplastic cells are arranged in ribbons or palisades (Fig. 4C). The fibrillary pattern of neoplastic tissue, resembling diffuse astrocytomas,

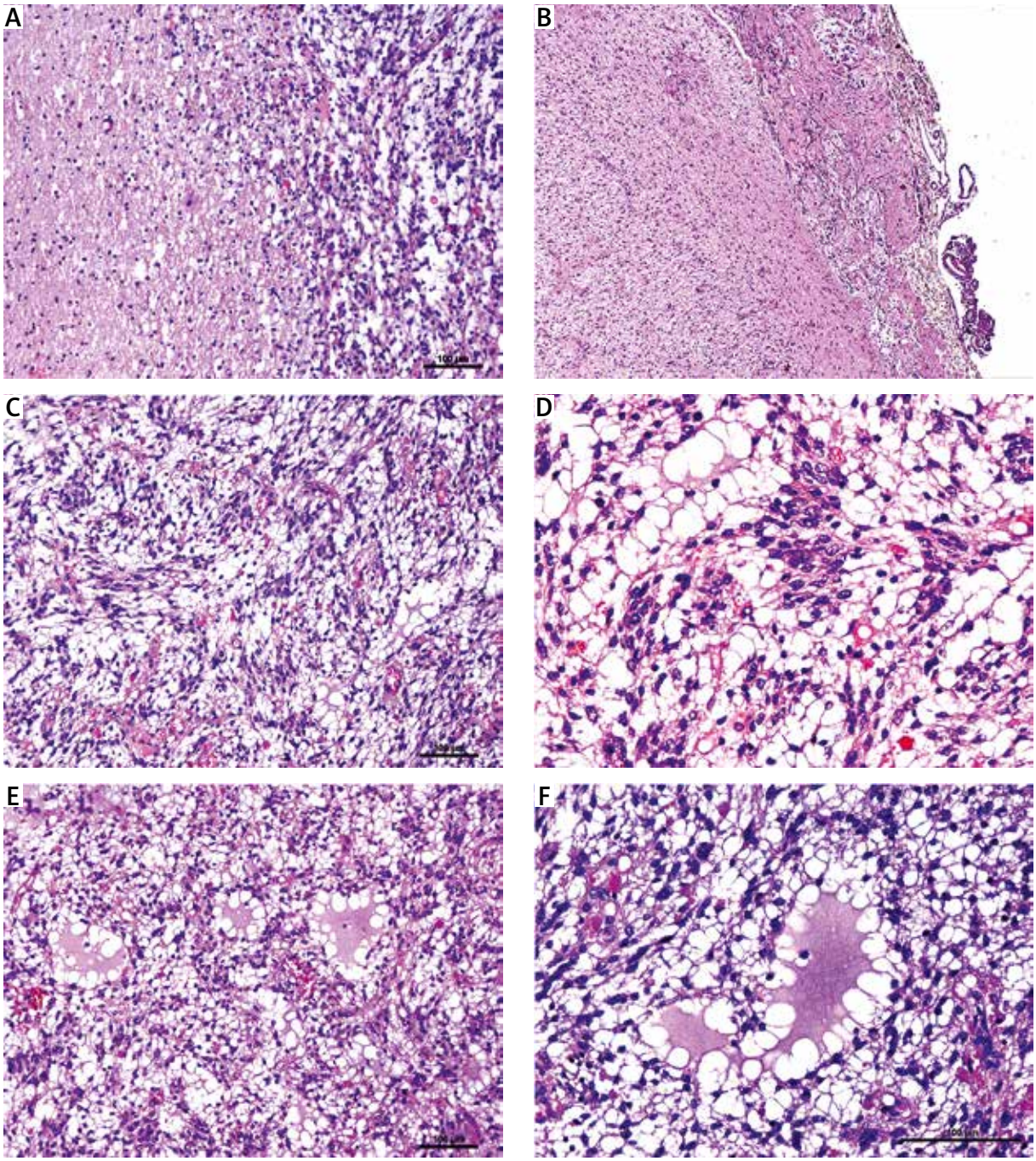


Fig. 1. Morphology of pilocytic astrocytoma. **A**) Well demarcation of the tumour from the surrounding tissue with only limited infiltration along the border zone. **B**) Superficially located tumour with leptomeningeal tumour growth. **C**) Classic biphasic pattern with microcystic tissue. **D**) Biphasic architecture with loose, spongy component and compact, fibrillary elements. **E**) Small cysts of different size and shape within loose, microcystic background. **F**) Microcyst filled with eosinophilic, myxoid material.

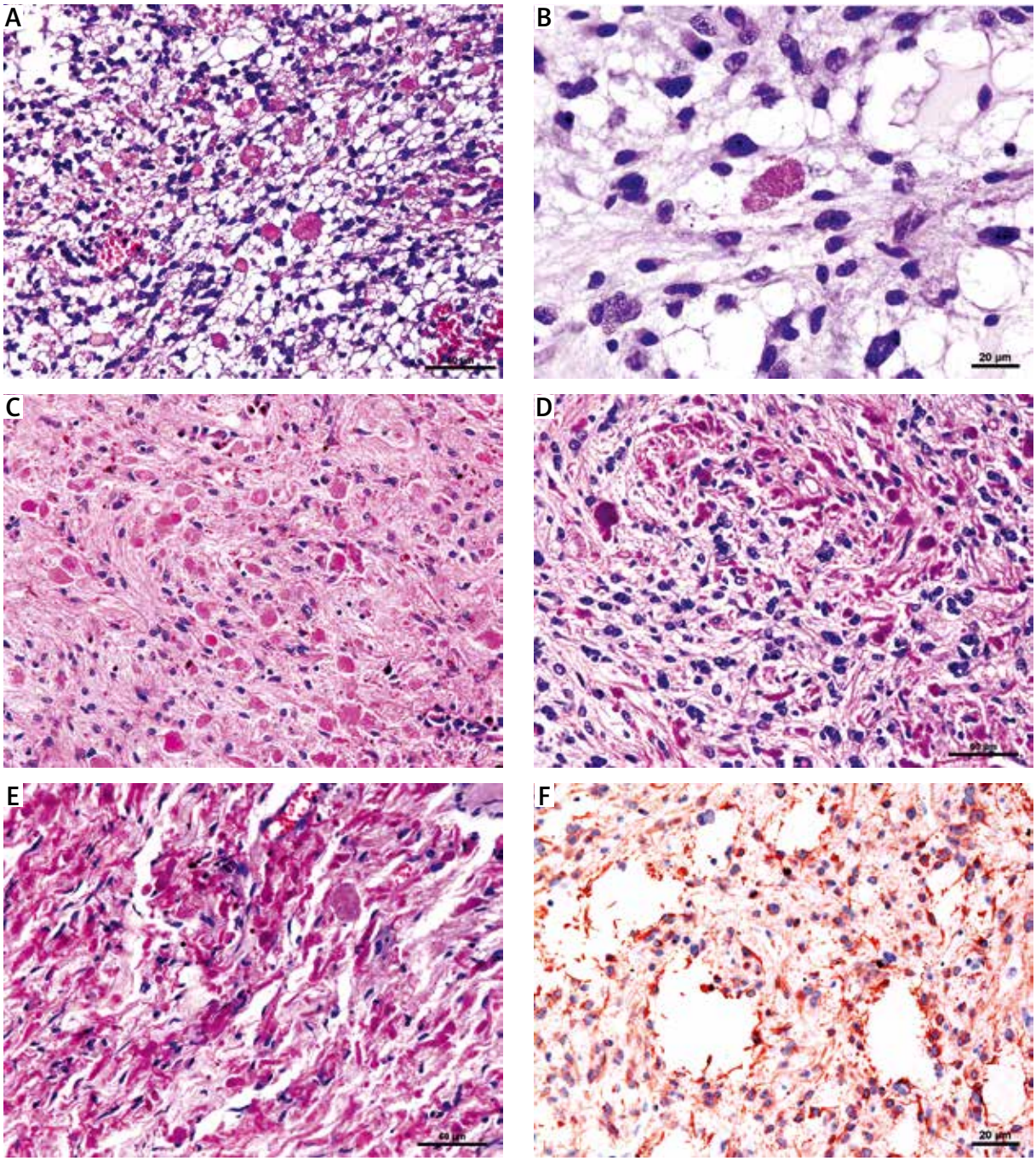


Fig. 2. Typical histopathological features of pilocytic astrocytoma. **A**) Microcystic, loose-textured areas containing eosinophilic granular bodies (EGBs) and hyaline droplets. **B**) Eosinophilic granular bodies formed by the aggregates of tiny granular bodies. **C**) Eosinophilic, compact region with numerous eosinophilic structures corresponding to EGBs and/or hyaline droplets. **D**) More solid component with increased cellularity and focal accumulation of Rosenthal fibres. **E**) Compact, fibrillary tumour tissue with numerous Rosenthal fibres. **F**) Glial fibrillary acidic protein expression in neoplastic cells.

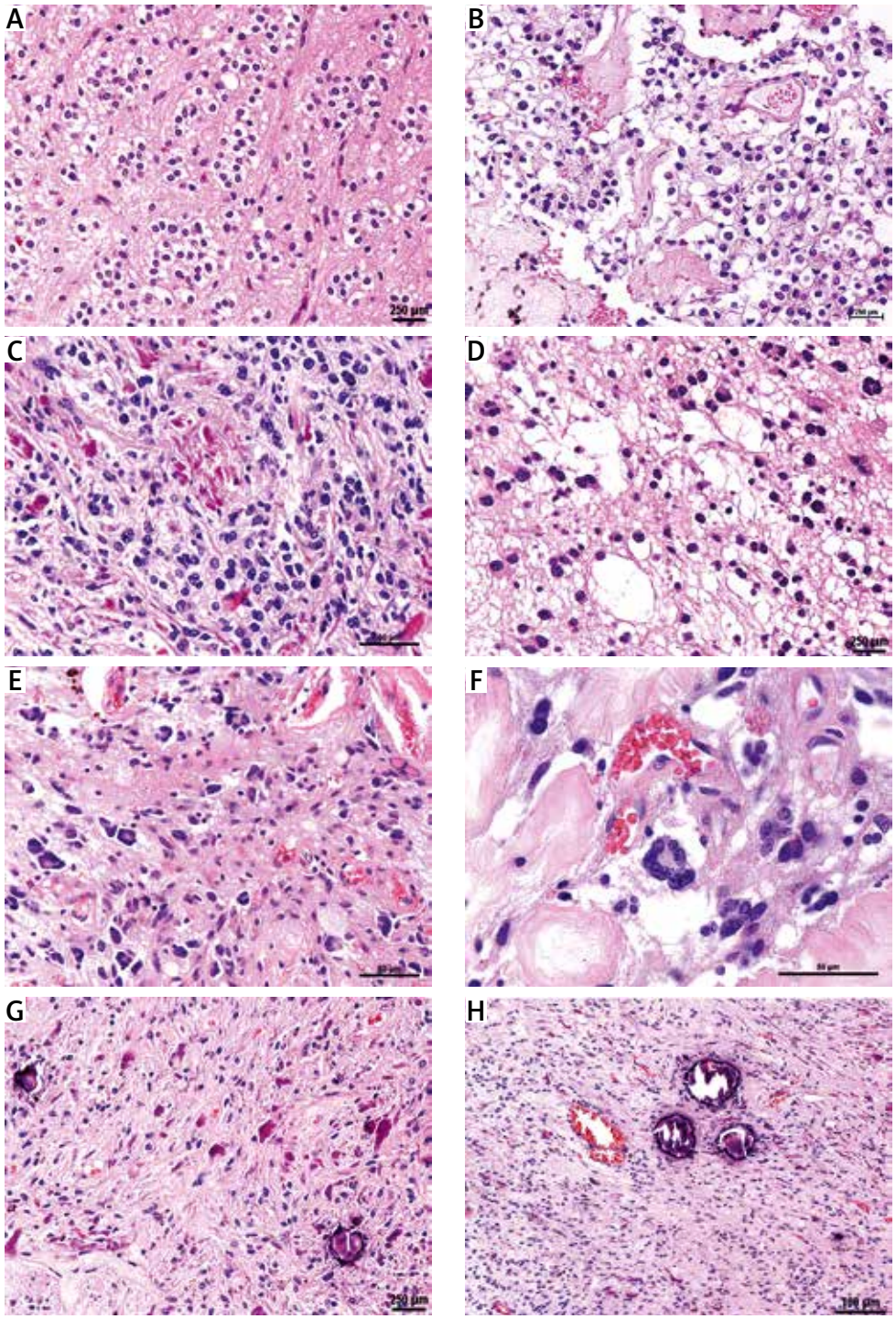


Fig. 3. Heterogeneous pattern of pilocytic astrocytoma. **A**) Pilocytic astrocytoma with predominant oligodendroglioma-like architecture. **B**) Oligodendroglioma-like pattern with uniform cells exhibiting clear perinuclear halo. **C**) Compact tissue with increased cellularity, nuclear hyperchromasia and atypia. Rosenthal fibres are present. **D**) Spongy tissue with pleomorphic cells displaying degenerative nuclear atypia. **E**) Large, pleomorphic, often multinucleated cells. **F**) Cell with multiple nuclei arranged circumferentially in the cytoplasm, corresponding to "pennies-on-a-plate". **G**) Compact, fibrillary tissue with Rosenthal fibres and tiny calcifications. **H**) Fibrillary tissue with psammomatous calcifications.

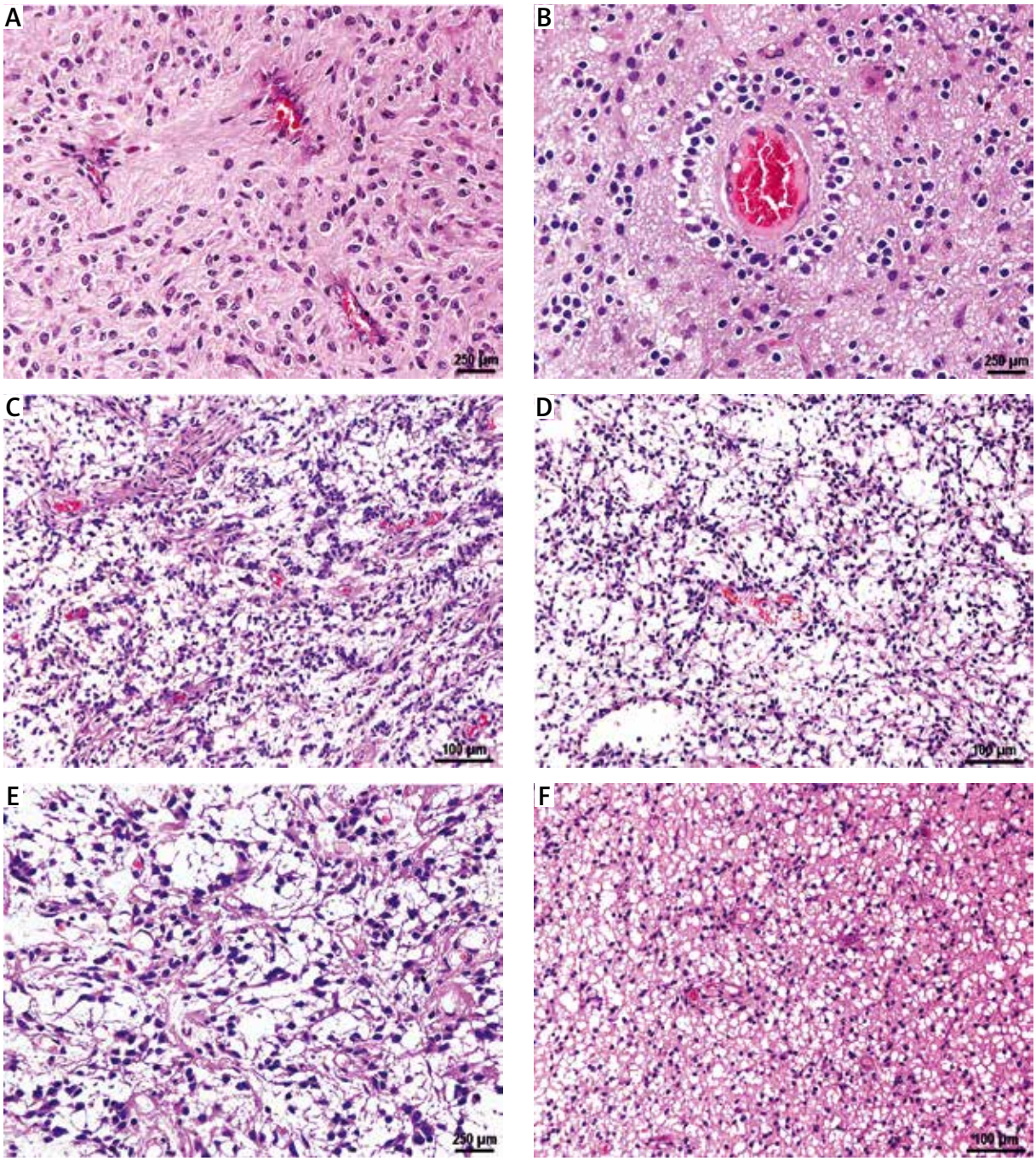


Fig. 4. Patterns of pilocytic astrocytoma mimicking other glial neoplasms. **A**) Ependymoma-like perivascular arrangement of neoplastic cells. **B**) Angiocentric arrangement of neoplastic cells resembling oligodendroglioma cells. **C**) Ribbons or palisaded arrangement of neoplastic cells. **D**) Tumour tissue resembling diffuse astrocytomas. **E**) Neoplastic cells with cytological atypia, nuclear hyperchromasia and fine fibrillary background. **F**) Slightly spongy tissue composed of uniform neoplastic fibrillary astrocytic cells mimicking diffuse astrocytoma.

is not unique (Fig. 4D). Sometimes, the fine fibrillary background is accompanied by cytological atypia, uneven cell distribution and nuclear hyperchromasia (Fig. 4E). Slightly spongy tissue composed of uniform neoplastic fibrillary astrocytic cells may mimic diffuse astrocytoma (Fig. 4F).

Pilocytic astrocytomas often appear as highly vascular tumours. The blood vessels may exhibit advanced fibrosis and/or hyalinization of vascular walls (Fig. 5A). Sometimes, the closely packed, sclerotic, largely hyalinized vessels can resemble vascular malformations, i.e. cavernous angioma (Fig. 5B). The neoplastic tissue may be scant and dispersed between conglomerates of thickened, hyalinized blood vessels (Fig. 5C, D), thus more mimicking reactive piloid gliosis around a lesion of vascular pathology than true neoplastic proliferation. In such cases the misleading diagnosis of cavernous angioma might be established. The advanced angiomatous proliferation might resemble capillary haemangioma, and the diagnosis of so-called angioglioma has been proposed [62]. In chronic lesions, the hemosiderin deposits of old haemorrhages and calcifications are often seen. An unusual case of pigmented cerebellar PA, presenting with posttraumatic haemorrhage in a 38-year-old man, has been described [93]. Advanced vascular abnormalities are sometimes associated with infarct-like necrosis. Other vascular changes are associated with proliferation of microvessels. Such microvascular proliferation often appears as linear arrays (Fig. 5E), especially within the cyst wall of the tumour. Typical glomeruloid changes of the vessels with hyperplasia of endothelial cells might suggest the diagnosis of high-grade gliomas (Fig. 5F). However, the microvascular proliferation of glomeruloid type in PAs should not be considered as a sign of anaplasia. Nevertheless, the careful analysis of clinicopathological findings supported by the molecular profile is required to distinguish PA from diffusely infiltrating gliomas.

Additionally, nuclear atypia, mitosis, necrosis, vascular proliferation and spread into leptomeninges and/or surrounding tissue, mimicking high-grade diffuse gliomas, may be rarely observed. Only occasionally, tumours with brisk mitotic activity, necrosis and marked nuclear pleomorphism have been diagnosed as anaplastic PA [82]. It has been documented that anaplastic/malignant transformation of PA occurred most often in association with previous ionizing radiation [75] or surgery alone [88], whereas spontaneous malignant transformation was unique [10,72,90]. The majority of anaplastic cases have been described in adults [10,101]. Nevertheless, PAs with anaplastic features do not always exhibit a clear correlation between morphology and tumour behaviour. Only some cases of malignant PA have been documented to progress extremely rapidly [53].

Occasionally, the pre-existing neurons may be entrapped in the neoplastic tissue. Such lesions with residual neuronal elements should be distinguished from true gangliogliomas with a piloid astroglial component.

Histological variant

The pilomyxoid variant (PMA) of pilocytic astrocytoma exhibits different histological features and behaves more aggressively than PA [50]. It demonstrates piloid cells within a markedly loose, myxoid background that lacks Rosenthal fibres or EGB, characteristic for classic PAs (Fig. 6A). Bipolar tumour cells are arranged radially around blood vessels, thus resembling the angiocentric arrangement or perivascular pseudorosettes seen in ependymomas (Fig. 6B). Some tumours revealed intermediate features of both PMA and classic PA.

Pilomyxoid astrocytoma usually develops in the hypothalamic region, optic chiasm and third ventricle in infants and young children, less commonly in the thalamus, posterior fossa, brain stem, temporal lobe and spinal cord [49]. It has been suggested that PMAs ought to be included in the differential diagnoses of the lesions invading the sella turcica [3].

The clinical behaviour of PMAs is unpredictable. Usually, they behave more aggressively than conventional PAs. Pilomyxoid astrocytoma was classified as a tumour of grade II in the 2007 WHO classification of tumours of the central nervous system [17], but in the current revised 4th edition of the WHO 2016 classification, the determination of its grade of malignancy is not recommended.

Immunohistochemistry

Pilocytic astrocytomas express markers from the astrocytic lineage. They show strong GFAP immunostaining, S-100 protein and OLIG2 positivity. The Rosenthal fibres are strongly positive for α-B crystallin and exhibit only peripheral GFAP staining. Synaptophysin immunoexpression could be found

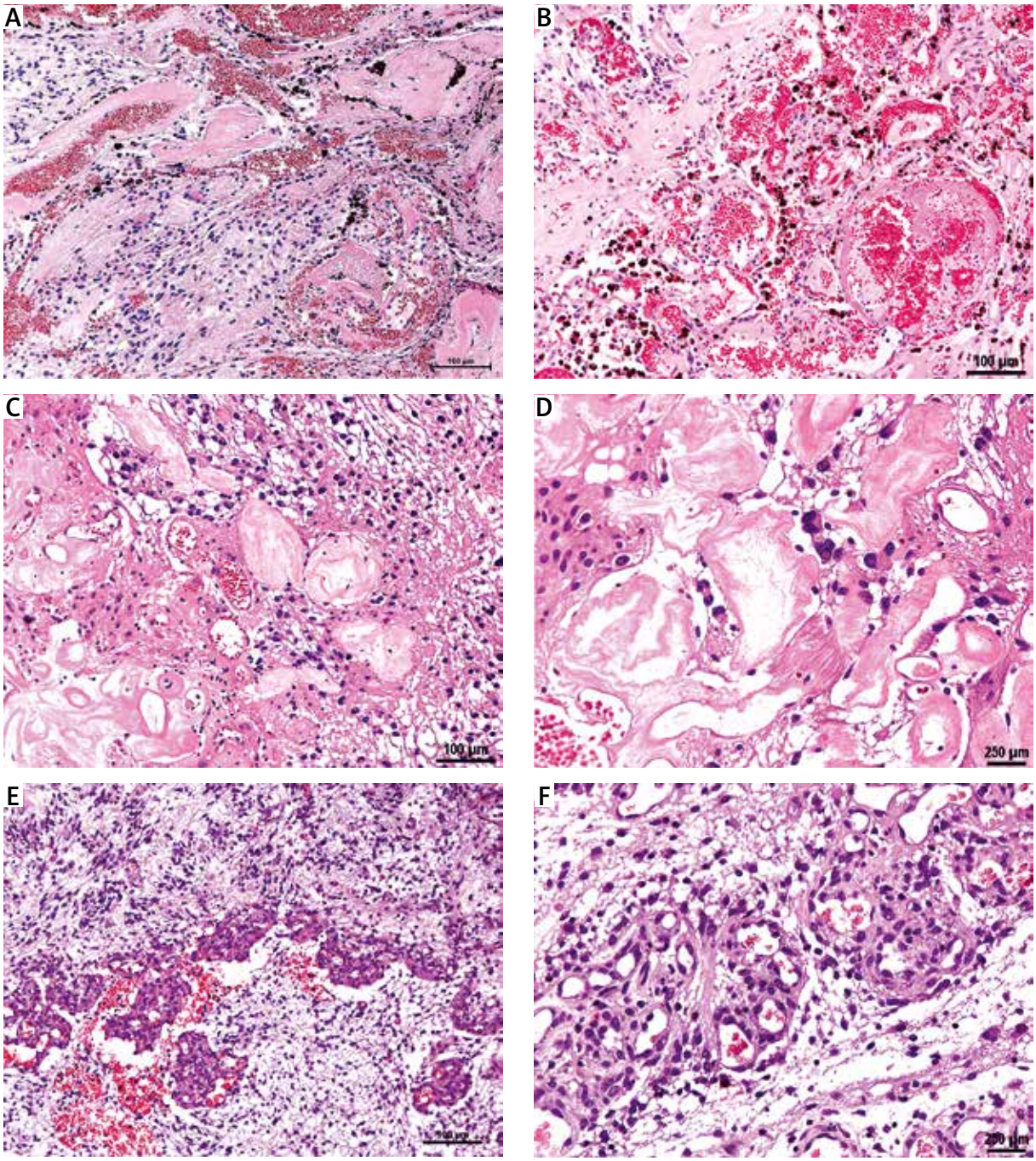


Fig. 5. Vascular changes in pilocytic astrocytomas. **A**) Neoplastic tissue with advanced vascular abnormalities associated with intratumoural bleeding. **B**) Conglomerates of hyalinized vessels and hemosiderin deposits. **C**) Vessels with considerably thickened, fibrous walls. **D**) Severe vascular hyalinization surrounded by a few neoplastic cells dispersed between conglomerates of vessels. **E**) Linear arrays of proliferating microvessels. **F**) Glomeruloid changes of the microvessels with hyperplasia of endothelial cells mimicking high-grade glioma.

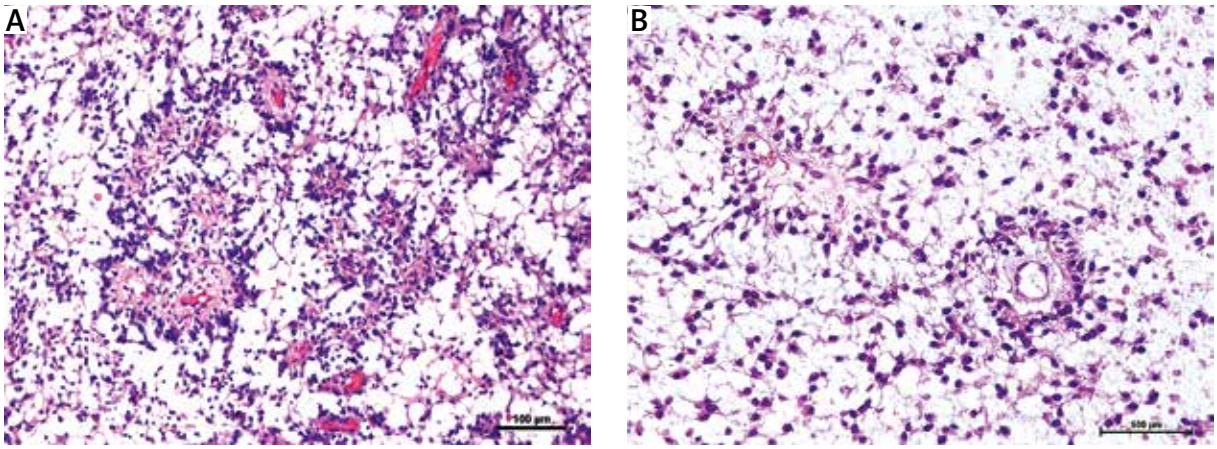


Fig. 6. Pilomyxoid astrocytoma. **A**) Markedly loose, myxoid background with angiocentric arrangement of neoplastic cells. **B**) Bipolar tumour cells arranged radially around blood vessels in loose, myxoid tissue.

focally. The Ki-67 proliferative index is usually low, sometimes with a focal increase. Immunohistochemistry for R132H-mutant IDH1 protein is absent in all PAs and helps to distinguish PA from diffuse astrocytomas. Unfortunately, the majority of paediatric diffuse astrocytomas also lack *IDH1* and/or *IDH2* mutations. Phosphorylated MAPK immunostaining with V600E-mutant BRAF positivity could be detected.

Molecular findings

There are some genetic alterations that are considered to underlie the development of PAs, with a key role of dysregulation/activation of the mitogen-activated protein kinase (MAPK) pathway, particularly through a tandem duplication leading to an oncogenic *BRAF* fusion gene [76,83,102]. Mitogen-activated protein kinase pathway abnormalities are found in the vast majority of PA cases; thus PA is considered as a one-pathway disease [24]. Recent studies have pointed towards the role of activation of the PI3K/AKT pathway in addition to MAPK/ERK signalling pathways in histologically anaplastic and biologically aggressive PA variants [81].

In sporadic PAs the most common genetic alteration is a tandem duplication at chromosome 7q34, which results in fusion between *KIAA1549* and *BRAF* genes. This tandem duplication is found in 60-94% of PAs and leads to downstream activation of the MEK/MAPK/ERK/p16 pathway [12,23,85]. The *KIAA1549–BRAF* fusion is more commonly encountered in PAs originating in the cerebellum [40]. These molecular alterations are more frequent in children than in adults [87]. However, their prognostic significance is unclear. *KIAA1549-BRAF* fusion causes a deletion of the amino-terminal domain of *BRAF* and constitutive activation of its kinase activity [44]. A proteomic study confirmed the predominance of the MAPK pathway for childhood PA and introduced novel findings regarding ERK-2 expression [6].

The genetic association between neurofibromatosis type 1 and PA is well known. Patients with NF1 demonstrate inactivation of the tumour suppressor gene *NF1*, which encodes neurofibromin (NF1), a protein involved in the MAPK and mTOR-pathway through RAS-RAF signalling. Loss of neurofibromin leads to an increase of the active form of Ras and negative regulation of the RAS/ERK pathway and activation of the mammalian target of rapamycin mTOR/AKT pathway [21]. The Ras/ERK pathway is also involved in cell differentiation responsible for slow tumour growth. Mechanisms of tumourigenesis in low-grade gliomas with and without NF1 are different, including the different signalling pathways and tumour microenvironment [38].

The molecular changes can be used as diagnostic markers [26]. *KIAA1549/BRAF* fusion is common in PA but infrequent in diffuse gliomas, whereas *IDH1* mutation is common in diffuse astrocytomas in adults and uncommon in PA. Such molecular differences can help to establish the correct diagnosis [51]. Moreover, molecular targeted therapy might be considered as a possible novel therapeutic treatment, particularly in patients suffering from tumours with an unfavourable anatomical location preventing their complete surgical resection.

Treatment

Cerebellar and superficial cerebral pilocytic tumours are usually surgically treated by gross total or subtotal resection. Deeply situated lesions can be only partially resected. Disseminated PA lesions are usually treated by multi-modal therapy, including surgical resection, chemotherapy, and radiotherapy with extended-field radiation [16]. It is noteworthy that PAs are generally indolent tumours and attention should be paid to avoiding aggressive therapy [80].

Prognosis

Pilocytic astrocytomas are typically well-circumscribed, slowly growing WHO grade I lesions that are generally associated with excellent prognosis, favourable long-term outcome or spontaneous regression with a 10-year survival rate of more than 95% cases. Total or even subtotal removal is an effective treatment in cerebellar and superficial cerebral pilocytic tumours [29]. The long-term functional outcome is generally favourable, in the absence of post-operative complications and brain stem involvement [2]. The surgery is usually followed by tumour stabilization even when the tumour is only partially resected and the patient does not receive complementary radiation. Deeply located lesions are usually associated with worse prognosis and risk for local recurrence. However, some superficial tumours might also exhibit an unexpected clinical course with recurrences.

Spontaneous involution of PA has been reported in children, particularly in cases associated with NF1 [19,33,36,56,73,84]. It has been documented that the optic pathway/hypothalamic gliomas, treated by subtotal resection, biopsy and radiotherapy or radiation alone, might stabilize or even completely disappear on serial MR images [39]. This phenomenon has rarely been observed in adults, especially without NF1 [11]. Nevertheless, postoperative MRI monitoring seems to be important both in adults and in children.

Clinical behaviour is often unpredictable, and PAs may recur locally after incomplete resection or disseminate to the leptomeninges, especially when they are located in the hypothalamic region [1]. It must be noted that recurrences of PA are not associated with histological malignant progression. Some data suggest that PAs in adult patients behave more aggressively than in children and might be associated with recurrences, progression and higher mortality [31,43,91,94,99]. The possibility of rapid tumour recurrence and malignant transformation indicate the need for careful post-operative follow-up for adult patients with PAs [30].

Additionally, dissemination into the subarachnoid space occurs in a small percentage of cases, most often related to local recurrences. Dissemination of cerebellar PAs can be associated with obstructive hydrocephalus leading to an unfavourable prognosis [32,41,42]. Leptomeningeal dissemination of the cerebellar tumours into the spinal cord might occur many years after primary surgery [45,66,78]; less commonly the meningeal seeding appears at the initial presentation [67]. Spread into the spinal meninges can be observed in PAs of the spinal cord [1,9] and optic chiasm [4,48]. Extremely rarely, spinal cord PA can spread via the cerebrospinal fluid pathway (CSF) to the cerebral meninges [69]. Metastases of juvenile PA to the left cerebellar tonsil and lumbosacral region were also reported in PA of the hypothalamic region [70]. Only occasionally, the tumour may exhibit multifocal recurrences and extensive dissemination [7]. An unusual case of recurrent supratentorial intraventricular PA with local recurrence and disseminations along the complete neuraxis in a short period of one year after primary surgery was described [77].

A particular clinicopathological presentation of PAs might be associated with fatal haemorrhages, which are most often subarachnoid (SAH) [34,46,54, 61,64]. Such tumour manifestation is less commonly observed in PMA [35,89]. The haemorrhages likely result from vascular abnormalities, often observed in PAs. Tumour haemorrhage should be considered in the differential diagnosis of spontaneous intracerebral haemorrhage [97].

Differential diagnosis

The correct diagnosis of PA and its differentiation from diffuse gliomas is important as it is related to different prognosis and specific therapy based on the molecular signal pathways. The diagnostic criteria include age of patients, MRI findings, tumour location and typical histology with biphasic picture, presence of piloid cells and degenerative changes with EGBs and Rosenthal fibres. The majority of cases meet these criteria, but some of them present unusual clinicopathological features, including uncommon location, older age group, misleading histological appearance or inadequate biopsy spec-

imens. Histologically, the diagnosis of PA can often be challenging. The microscopic pictures of PA may mimic some other neoplasms, including pleomorphic xanthoastrocytoma, diffuse astrocytoma and glioblastoma or even piloid reactive gliosis typical for long-standing, non-neoplastic pathological processes. Distinction from all these lesions bears important therapeutic and prognostic implications. Caution is required to avoid misinterpretation; however, in small and/or non-representative biopsy specimens it can be difficult or even impossible. The frequent mutation of *IDH1* and *IDH2* in diffuse gliomas, which is usually not seen in PAs [51], allows tumours of different prognosis and treatment to be distinguished. Only one case of a 72-year-old woman with a right cerebellar PA of WHO grade I and *IDH1* mutation has been reported so far [13].

Conclusions

Pilocytic astrocytoma is a tumour of distinct clinicopathological features, but not infrequently it presents with atypical features, including older age of patients, uncommon location and lack of classic biphasic morphology. The different histopathological pattern, presence of nuclear atypia, vascular changes, mitoses, necrosis and glomeruloid microvascular proliferation can lead to misdiagnosis. Moreover, the biological behaviour of PAs independently of their morphology is not always predictable. In conclusion, the diagnosis of PA may be challenging, and careful analysis of clinical, histopathological and molecular features is required to avoid misinterpretation followed by inadequate therapy.

Disclosure

Authors report no conflict of interest.

References

1. Abel TJ, Chowdhary A, Thapa M, Rutledge JC, Geyer JR, Ojemann J, Avellino AM. Spinal cord pilocytic astrocytoma with leptomeningeal dissemination to the brain. Case report and review of the literature. J Neurosurg 2006; 105: 508-514.
2. Ait Khelifa-Gallois N, Laroussinie F, Puget S, Sainte-Rose C, Dellatolas G. Long-term functional outcome of patients with cerebellar pilocytic astrocytoma surgically treated in childhood. Brain Inj 2015; 29: 366-373.
3. Alimohamadi M, Bidabadi MS, Ayan Z, Ketabchi E, Amirjamshidi A. Pilomyxoid astrocytoma with involvement of the sella turcica in an adolescent. J Clin Neurosci 2009; 16: 1648-1649.
4. Alyeldien A, Teuber-Hanselmann S, Cheko A, Holl T, Scholz M, Petridis AK. Diffuse Spinal Leptomeningeal Spread of a Pilocytic Astrocytoma in a 3-year-old Child. Clin Pract 2016; 6: 813.
5. Amato VG, Arienta C, Sparacio F. Dorsally exophytic brain stem tumors: total removal of a medullary pilocytic astrocytoma in child. Clinicopathological considerations and case report. J Neurosurg Sci 1999; 43: 299-304.
6. Anagnostopoulos AK, Dimas KS, Papathanassiou C, Braoudaki M, Anastasiadou E, Vougas K, Karamolegou K, Kontos H, Prodromou N, Tzortzatou-Stathopoulou F, Tsangaris GT. Proteomics studies of childhood pilocytic astrocytoma. J Proteome Res 2011; 10: 2555-2565.
7. Andrychowski J, Taraszewska A, Czernicki Z, Jurkiewicz J, Netczuk T, Dabrowski P. Ten years observation and treatment of multifocal pilocytic astrocytoma. Folia Neuropathol 2009; 47: 362-370.
8. Arantes M, Vaz AR, Honavar M, Resende M, Pereira JR. Pilocytic astrocytoma arising from the pineal gland. J Neuroradiol 2009; 36: 177-178.
9. Arulrajah S, Huisman TA. Pilomyxoid astrocytoma of the spinal cord with cerebrospinal fluid and peritoneal metastasis. Neuropediatrics 2008; 39: 243-245.
10. Azad A, Deb S, Cher L. Primary anaplastic pilocytic astrocytoma. J Clin Neurosci 2009; 16: 1704-1706.
11. Balkhoyor KB, Bernstein M. Involution of diencephalic pilocytic astrocytoma after partial resection. Report of two cases in adults. J Neurosurg 2000; 93: 484-486.
12. Bar EE, Lin A, Tihan T, Burger PC, Eberhart CG. Frequent gains at chromosome 7q34 involving BRAF in pilocytic astrocytoma. J Neuropathol Exp Neurol 2008; 67: 878-887.
13. Behling F, Steinhilber J, Tatagiba M, Bisdas S, Schittenhelm J. IDH1 R132H mutation in a pilocytic astrocytoma: a case report. Int J Clin Exp Pathol 2015; 8: 11809-11813.
14. Bell D, Chitnavis BP, Al-Sarraj S, Connor S, Sharr MM, Gullan RW. Pilocytic astrocytoma of the adult--clinical features, radiological features and management. Br J Neurosurg 2004; 18: 613-616.
15. Berhouma M, Jemel H, Kchir N. Calcified pilocytic astrocytoma of the medulla mimicking a brainstem "stone". Pathologica 2008; 100: 408-410.
16. Bian SX, McAleer MF, Vats TS, Mahajan A, Grosshans DR. Pilocytic astrocytoma with leptomeningeal dissemination. Childs Nerv Syst 2013; 29: 441-450.
17. Brat DJ, Scheithauer BW, Fuller GN, Tihan T. Newly codified glial neoplasms of the 2007 WHO Classification of Tumours of the Central Nervous System: angiocentric glioma, pilomyxoid astrocytoma and pituicytoma. Brain Pathol 2007; 17: 319-324.
18. Brown PD, Anderson SK, Carrero XW, O'Neill BP, Giannini C, Galanis E, Shah SA, Abrams RA, Curran WJ Jr, Buckner JC, Shaw EG. Adult patients with supratentorial pilocytic astrocytoma: long-term follow-up of prospective multicenter clinical trial NCCTG-867251 (Alliance). Neurooncol Pract 2015; 2: 199-204.
19. Brzowski AE, Bazan C 3rd, Mumma JV, Ryan SG. Spontaneous regression of optic glioma in a patient with neurofibromatosis. Neurology 1992; 42: 679-681.

20. Burkhardt K, Heuberger F, Delavelle J. Pilocytic astrocytoma in the elderly. Clin Neuropathol 2007; 26: 306-310.
21. Chen YH, Gutmann DH. The molecular and cell biology of pediatric low-grade gliomas. Oncogene 2014; 33: 2019-2026.
22. Chourmouzi D, Papadopoulou E, Konstantinidis M, Syrris V, Kouskouras K, Haritanti A, Karkavelas G, Drevelegas A. Manifestations of pilocytic astrocytoma: a pictorial review. Insights Imaging 2014; 5: 387-402.
23. Cin H, Meyer C, Herr R, Janzarik WG, Lambert S, Jones DT, Jacob K, Benner A, Witt H, Remke M, Bender S, Falkenstein F, Van Anh TN, Olbrich H, von Deimling A, Pekrun A, Kulozik AE, Gnekow A, Scheurlen W, Witt O, Omran H, Jabado N, Collins VP, Brummer T, Marschalek R, Lichter P, Korshunov A, Pfister SM. Oncogenic FAM131B-BRAF fusion resulting from 7q34 deletion comprises an alternative mechanism of MAPK pathway activation in pilocytic astrocytoma. Acta Neuropathol 2011; 121: 763-774.
24. Collins VP, Jones DT, Giannini C. Pilocytic astrocytoma: pathology, molecular mechanisms and markers. Acta Neuropathol 2015; 129: 775-788.
25. Collins VP TT, VanderBerg SR, Burger PC, Hawkins C. Pilocytic astrocytoma. In: WHO Classification of Tumors of the Central Nervous System. Louis DN, Oghaki H, Wiesttler OD, Cavenee WK, Elison DW (eds.). 4th ed. IARC Press, Lyon 2016; pp. 80-89.
26. Cykowski MD, Allen RA, Kanaly AC, Fung KM, Marshall R, Perry A, Stolzenberg ED, Dunn ST. The differential diagnosis of pilocytic astrocytoma with atypical features and malignant glioma: an analysis of 16 cases with emphasis on distinguishing molecular features. J Neurooncol 2013; 115: 477-486.
27. Cyrine S, Sonia Z, Mounir T, Badderedine S, Kalthoum T, Hedi K, Moncef M. Pilocytic astrocytoma: a retrospective study of 32 cases. Clin Neurol Neurosurg 2013; 115: 1220-1225.
28. Daszkiewicz P, Maryniak A, Roszkowski M, Barszcz S. Long-term functional outcome of surgical treatment of juvenile pilocytic astrocytoma of the cerebellum in children. Childs Nerv Syst 2009; 25: 855-860.
29. Dodgshun AJ, Maixner WJ, Hansford JR, Sullivan MJ. Low rates of recurrence and slow progression of pediatric pilocytic astrocytoma after gross-total resection: justification for reducing surveillance imaging. J Neurosurg Pediatr 2016; 17: 569-572.
30. Ellis JA, Waziri A, Balmaceda C, Canoll P, Bruce JN, Sisti MB. Rapid recurrence and malignant transformation of pilocytic astrocytoma in adult patients. J Neurooncol 2009; 95: 377-382.
31. Fiechter M, Hewer E, Knecht U, Wiest R, Beck J, Raabe A, Oertel MF. Adult anaplastic pilocytic astrocytoma – a diagnostic challenge? A case series and literature review. Clin Neurol Neurosurg 2016; 147: 98-104.
32. Figueiredo EG, Matushita H, Machado AG, Plese JP, Rosemberg S, Marino R, Jr. Leptomeningeal dissemination of pilocytic astrocytoma at diagnosis in childhood: two cases report. Arq Neuropsiquiatr 2003; 61: 842-847.
33. Gallucci M, Catalucci A, Scheithauer BW, Forbes GS. Spontaneous involution of pilocytic astrocytoma in a patient without neurofibromatosis type 1: case report. Radiology 2000; 214: 223-226.
34. Garg A, Chugh M, Gaikwad SB, Chandra SP, Gupta V, Mishra NK, Sharma MC. Juvenile pilocytic astrocytoma presenting with subarachnoid hemorrhage. Case report and review of the literature. J Neurosurg 2004; 100: 525-529.
35. Gottfried ON, Fults DW, Townsend JJ, Couldwell WT. Spontaneous hemorrhage associated with a pilomyxoid astrocytoma. Case report. J Neurosurg 2003; 99: 416-420.
36. Gottschalk S, Tavakolian R, Buske A, Tinschert S, Lehmann R. Spontaneous remission of chiasmatic/hypothalamic masses in neurofibromatosis type 1: report of two cases. Neuroradiology 1999; 41: 199-201.
37. Harraher CD, Vogel H, Steinberg GK. Spinal pilocytic astrocytoma in an elderly patient. World Neurosurg 2013; 79: 799 E797-799.
38. Helfferich J, Nijmeijer R, Brouwer OF, Boon M, Fock A, Hoving EW, Meijer L, den Dunnen WF, de Bont ES. Neurofibromatosis type 1 associated low grade gliomas: A comparison with sporadic low grade gliomas. Crit Rev Oncol Hematol 2016; 104: 30-41.
39. Hoffman HJ, Humphreys RP, Drake JM, Rutka JT, Becker LE, Jenkin D, Greenberg M. Optic pathway/hypothalamic gliomas: a dilemma in management. Pediatr Neurosurg 1993; 19: 186-195.
40. Horbinski C, Hamilton RL, Nikiforov Y, Pollack IF. Association of molecular alterations, including BRAF, with biology and outcome in pilocytic astrocytomas. Acta Neuropathol 2010; 119: 641-649.
41. Jamjoom AB, Jamjoom ZA, al-Rayess M. Intraventricular and leptomeningeal dissemination of a pilocytic cerebellar astrocytoma in a child with a ventriculoperitoneal shunt: case report. Br J Neurosurg 1998; 12: 56-58.
42. Jandaghi AB, Bidabadi E, Saadat S, Alijani B, Daliri S, Reyhanian Z, Mashouf M. Leptomeningeal dissemination of pilocytic astrocytoma in a 17-year-old boy. Turk Neurosurg 2014; 24: 978-981.
43. Johnson DR, Brown PD, Galanis E, Hammack JE. Pilocytic astrocytoma survival in adults: analysis of the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute. J Neurooncol 2012; 108: 187-193.
44. Jones DT, Gronych J, Lichter P, Witt O, Pfister SM. MAPK pathway activation in pilocytic astrocytoma. Cell Mol Life Sci 2012; 69: 1799-1811.
45. Kanda M, Tanaka H, Shinoda S, Masuzawa T. Leptomeningeal dissemination of pilocytic astrocytoma via hematoma in a child. Case report. Neurosurg Focus 2002; 13: ECP2.
46. Kato K, Moteki Y, Nakagawa M, Kadoyama S, Ujiie H. Subarachnoid hemorrhage caused by pilocytic astrocytoma – case report. Neurol Med Chir (Tokyo) 51: 82-84.
47. Kim YE, Shin HJ, Suh YL. Pilocytic astrocytoma with extensive psammomatous calcification in the lateral ventricle: a case report. Childs Nerv Syst 2012; 28: 649-652.
48. Kocks W, Kalff R, Reinhardt V, Grote W, Hilke J. Spinal metastasis of pilocytic astrocytoma of the chiasma opticum. Childs Nerv Syst 1989; 5: 118-120.
49. Komotar RJ, Mocco J, Carson BS, Sughrue ME, Zacharia BE, Sisti AC, Canoll PD, Khandji AG, Tihan T, Burger PC, Bruce JN. Pilomyxoid astrocytoma: a review. MedGenMed 2004; 6: 42.
50. Komotar RJ, Mocco J, Jones JE, Zacharia BE, Tihan T, Feldstein NA, Anderson RC. Pilomyxoid astrocytoma: diagnosis, prognosis, and management. Neurosurg Focus 2005; 18: E7.

51. Korshunov A, Meyer J, Capper D, Christians A, Remke M, Witt H, Pfister S, von Deimling A, Hartmann C. Combined molecular analysis of BRAF and IDH1 distinguishes pilocytic astrocytoma from diffuse astrocytoma. Acta Neuropathol 2009; 118: 401-405.
52. Kumar R, Garg K, Kakkar A, Sharma MC. Giant calcified intraventricular pilocytic astrocytoma: a rare entity. Neurol India 2014; 62: 71-73.
53. Kuroiwa T, Ohta T, Tsutsumi A. Malignant pilocytic astrocytoma in the medulla oblongata: case report. Brain Tumor Pathol 1999; 16: 81-85.
54. Lee CS, Huh JS, Sim KB, Kim YW. Cerebellar pilocytic astrocytoma presenting with intratumor bleeding, subarachnoid hemorrhage, and subdural hematoma. Childs Nerv Syst 2009; 25: 125-128.
55. Lee YY, Van Tassel P, Bruner JM, Moser RP, Share JC. Juvenile pilocytic astrocytomas: CT and MR characteristics. AJR Am J Roentgenol 1989; 152: 1263-1270.
56. Leisti EL, Pyhtinen J, Poyhonen M. Spontaneous decrease of a pilocytic astrocytoma in neurofibromatosis type 1. AJNR Am J Neuroradiol 1996; 17: 1691-1694.
57. Li HM, Hsu SS, Wang JS, Weng MJ, Fu JH, Chen CK, Lai PH. Cerebral pilocytic astrocytoma with spontaneous intracranial hemorrhage in adults. J Chin Med Assoc 2008; 71: 587-593.
58. Listernick R, Ferner RE, Liu GT, Gutmann DH. Optic pathway gliomas in neurofibromatosis-1: controversies and recommendations. Ann Neurol 2007; 61: 189-198.
59. Lyons MK. Pilocytic astrocytoma with spontaneous intracranial hemorrhages in an elderly adult. Clin Neurol Neurosurg 2007; 109: 76-80.
60. Malik A, Deb P, Sharma MC, Sarkar C. Neuropathological spectrum of pilocytic astrocytoma: an Indian series of 120 cases. Pathol Oncol Res 2006; 12: 164-171.
61. Matsumoto K, Akagi K, Abekura M, Maeda Y, Kitagawa M, Ryujin H, Iwasa N. Hypothalamic pilocytic astrocytoma presenting with intratumoral and subarachnoid hemorrhage. Neurol Med Chir (Tokyo) 1997; 37: 849-851.
62. Matyja E, Grajkowska W, Taraszewska A, Marchel A, Bojarski P, Nauman P. Advanced reactive astrogliosis associated with hemangioblastoma versus astroglial-vascular neoplasm ("angioglioma"). Folia Neuropathol 2007; 45: 120-125.
63. Medress ZA, Xu LW, Ziskin JL, Lefterova MI, Vogel H, Li G. Pilocytic astrocytoma with IDH1 mutation in the cerebellum of an elderly patient. Clin Neuropathol 2015; 34: 96-98.
64. Mesiwala AH, Avellino AM, Roberts TS, Ellenbogen RG. Spontaneous cerebellar hemorrhage due to a juvenile pilocytic astrocytoma: case report and review of the literature. Pediatr Neurosurg 2001; 34: 235-238.
65. Mirone G, Schiabello L, Chibbaro S, Bouazza S, George B. Pediatric primary pilocytic astrocytoma of the cerebellopontine angle: a case report. Childs Nerv Syst 2009; 25: 247-251.
66. Mishima K, Nakamura M, Nakamura H, Nakamura O, Funata N, Shitara N. Leptomeningeal dissemination of cerebellar pilocytic astrocytoma. Case report. J Neurosurg 1992; 77: 788-791.
67. Morikawa M, Tamaki N, Kokunai T, Nagashima T, Kurata H, Yamamoto K, Imai Y, Itoh H. Cerebellar pilocytic astrocytoma with leptomeningeal dissemination: case report. Surg Neurol 1997; 48: 49-51; discussion 51-42.
68. Nakano Y, Yamamoto J, Takahashi M, Soejima Y, Akiba D, Kitagawa T, Ueta K, Miyaoka R, Umemura T, Nishizawa S. Pilocytic astrocytoma presenting with atypical features on magnetic resonance imaging. J Neuroradiol 2015; 42: 278-282.
69. Ng HK, Leung CH, Boet R, Poon WS. Spinal cord pilocytic astrocytoma with cranial meningeal metastases. J Clin Neurosci 2001; 8: 374-377.
70. Obana WG, Cogen PH, Davis RL, Edwards MS. Metastatic juvenile pilocytic astrocytoma. Case report. J Neurosurg 1991; 75: 972-975.
71. Ostrom QT, Gittleman H, Liao P, Rouse C, Chen Y, Dowling J, Wolinsky Y, Kruchko C, Barnholtz-Sloan J. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2007-2011. Neuro Oncol 2014; 16 Suppl 4: iv1-63.
72. Otero-Rodriguez A, Sarabia-Herrero R, Garcia-Tejeiro M, Zamora-Martinez T. Spontaneous malignant transformation of a supratentorial pilocytic astrocytoma. Neurocirugia (Astur) 2010; 21: 245-252.
73. Parazzini C, Triulzi F, Bianchini E, Agnetti V, Conti M, Zanolini C, Maninetti MM, Rossi LN, Scotti G. Spontaneous involution of optic pathway lesions in neurofibromatosis type 1: serial contrast MR evaluation. AJNR Am J Neuroradiol 1995; 16: 1711-1718.
74. Pasarikovski CR, Allevato PA, Hammond R, Elashaal AA. Pilocytic astrocytoma of the lower thoracic spine in an adult patient. Acta Neurochir (Wien) 2015; 157: 1179-1181.
75. Peters KB, Cummings TJ, Gururangan S. Transformation of juvenile pilocytic astrocytoma to anaplastic pilocytic astrocytoma in patients with neurofibromatosis type I. J Pediatr Hematol Oncol 2011; 33: e198-201.
76. Pfister S, Janzarik WG, Remke M, Ernst A, Werft W, Becker N, Toedt G, Wittmann A, Kratz C, Olbrich H, Ahmadi R, Thieme B, Joos S, Radlwimmer B, Kulozik A, Pietsch T, Herold-Mende C, Gnekow A, Reifenberger G, Korshunov A, Scheurlen W, Omran H, Lichter P. BRAF gene duplication constitutes a mechanism of MAPK pathway activation in low-grade astrocytomas. J Clin Invest 2008; 118: 1739-1749.
77. Raheja A, Singh PK, Nambirajan A, Sharma MC, Sharma BS. Diffuse leptomeningeal spread of supratentorial recurrent pilocytic astrocytoma in a child. J Pediatr Neurosci 2015; 10: 408-411.
78. Redhu R, Nadkarni TD. Multicompartmental cerebrospinal fluid dissemination of cerebellar pilocytic astrocytoma at presentation. J Clin Neurosci 2011; 18: 1412-1414.
79. Reis A, Kuzeyli K, Cobanoglu U, Cakir E, Usul H, Sari A. Pilocytic astrocytoma of neurohypophysis. Neuropathology 2003; 23: 214-218.
80. Reis GF, Tihan T. Therapeutic targets in pilocytic astrocytoma based on genetic analysis. Semin Pediatr Neurol 2015; 22: 23-27.
81. Rodriguez EF, Scheithauer BW, Giannini C, Rynearson A, Cen L, Hoesley B, Gilmer-Flynn H, Sarkaria JN, Jenkins S, Long J, Rodriguez FJ. PI3K/AKT pathway alterations are associated with clinically aggressive and histologically anaplastic subsets of pilocytic astrocytoma. Acta Neuropathol 2011; 121: 407-420.

82. Rodriguez FJ, Scheithauer BW, Burger PC, Jenkins S, Giannini C. Anaplasia in pilocytic astrocytoma predicts aggressive behavior. Am J Surg Pathol 2010; 34: 147-160.
83. Rodriguez FJ, Ligon AH, Horkayne-Szakaly I, Rushing EJ, Ligon KL, Vena N, Garcia DI, Cameron JD, Eberhart CG. BRAF duplications and MAPK pathway activation are frequent in gliomas of the optic nerve proper. J Neuropathol Exp Neurol 2012; 71: 789-794.
84. Rossi LN, Triulzi F, Parazzini C, Maninetti MM. Spontaneous improvement of optic pathway lesions in children with neurofibromatosis type 1. Neuropediatrics 1999; 30: 205-209.
85. Roth JJ, Santi M, Pollock AN, Harding BN, Rorke-Adams LB, Tooke LS, Biegel JA. Chromosome band 7q34 deletions resulting in KIAA1549-BRAF and FAM131B-BRAF fusions in pediatric low-grade Gliomas. Brain Pathol 2015; 25: 182-192.
86. Saad A, Tuli S, Ali EN, Houtchens M, Delalle I, Kesari S. Pilocytic astrocytoma of the spinal cord in an adult. J Neurooncol 2008; 88: 189-191.
87. Sadighi Z, Slopis J. Pilocytic astrocytoma: a disease with evolving molecular heterogeneity. J Child Neurol 2013; 28: 625-632.
88. Saito K, Toda M, Yoshida K. Pilocytic astrocytoma with anaplastic features presenting good long-term clinical course after surgery alone: a case report. Childs Nerv Syst 2015; 31: 167-171.
89. Shibahara I, Kanamori M, Kumabe T, Endo H, Sonoda Y, Ogawa Y, Watanabe M, Tominaga T. Hemorrhagic onset of pilocytic astrocytoma and pilomyxoid astrocytoma. Brain Tumor Pathol 2009; 26: 1-5.
90. Shibahara I, Kawaguchi T, Kanamori M, Yonezawa S, Takazawa H, Asano K, Ohkuma H, Kaimori M, Sasaki T, Nishijima M. Pilocytic astrocytoma with histological malignant features without previous radiation therapy – case report. Neurol Med Chir (Tokyo) 2011; 51: 144-147.
91. Stuer C, Vilz B, Majores M, Becker A, Schramm J, Simon M. Frequent recurrence and progression in pilocytic astrocytoma in adults. Cancer 2007; 110: 2799-2808.
92. Takada Y, Ohno K, Tamaki M, Hirakawa K. Cerebellopontine angle pilocytic astrocytoma mimicking acoustic schwannoma. Neuroradiology 1999; 41: 949-950.
93. Taraszewska A, Czernicki Z, Andrychowski J. Hemosiderin pigmentation of tumour cells in cerebellar pilocytic astrocytoma associated with post-traumatic hemorrhage in adults. Folia Neuropathol 2005; 43: 172-177.
94. Trabelsi S, Mama N, Ladib M, Popov S, Burford A, Mokni M, Tlili K, Krifa H, Varella-Garcia M, Jones C, Tahar Yacoubi M, Saad A, H'mida Ben Brahim D. Adult recurrent pilocytic astrocytoma: Clinical, histopathological and molecular study. Neurochirurgie 2015; 61: 392-397.
95. Utsuki S, Oka H, Kijima C, Yasui Y, Fujii K, Kawano N. Pilocytic astrocytoma with abundant oligodendroglioma-like component. Brain Tumor Pathol 2012; 29: 103-106.
96. Wade A, Hayhurst C, Amato-Watkins A, Lammie A, Leach P. Cerebellar pilocytic astrocytoma in adults: a management paradigm for a rare tumour. Acta Neurochir (Wien) 2013; 155: 1431-1435.
97. White JB, Piepgras DG, Scheithauer BW, Parisi JE. Rate of spontaneous hemorrhage in histologically proven cases of pilocytic astrocytoma. J Neurosurg 2008; 108: 223-226.
98. Xia J, Yin B, Liu L, Lu Y, Geng D, Tian W. Imaging Features of Pilocytic Astrocytoma in Cerebral Ventricles. Clin Neuroradiol 2015 [Epub ahead of print].
99. Ye JM, Ye MJ, Kranz S, Lo P. A 10 year retrospective study of surgical outcomes of adult intracranial pilocytic astrocytoma. J Clin Neurosci 2014; 21: 2160-2164.
100. Yeo YH, Byrne NP, Counelis GJ, Perry A. Adult with cerebellar anaplastic pilocytic astrocytoma associated with BRAF V600E mutation and p16 loss. Clin Neuropathol 2013; 32: 159-164.
101. Yong EX, McKelvie P, Murphy M, Wang YY. Anaplastic pilocytic astrocytoma. J Clin Neurosci 2014; 21: 1993-1996.
102. Yu J, Deshmukh H, Gutmann RJ, Emnett RJ, Rodriguez FJ, Watson MA, Nagarajan R, Gutmann DH. Alterations of BRAF and HIPK2 loci predominate in sporadic pilocytic astrocytoma. Neurology 2009; 73: 1526-1531.
103. Zhao Y, Feng L, Wei Q, Gao L. Adult cervicomedullary pilocytic astrocytoma: A case report. Experimental and therapeutic medicine 2015; 10: 2221-2223.

Review paper

Folia Neuropathologica

Sirtuins and their interactions with transcription factors and poly(ADP-ribose) polymerases

Henryk Jęśko[1], Robert P. Strosznajder[2]

[1]Department of Cellular Signalling, Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, [2]Laboratory of Preclinical Research and Environmental Agents, Department of Neurosurgery, Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland

Folia Neuropathol 2016; 54 (3): 212-233 DOI: 10.5114/fn.2016.62531

Abstract

Sirtuins (SIRT1 to -7) are unique histone deacetylases (HDACs) whose activity depends on NAD^+, thus making them capable of sensing the cellular metabolic status. Sirtuins orchestrate the stress response and damage repair, and are able to modulate the course of ageing and neurodegenerative diseases. Despite their classification as HDACs, sirtuins deacetylate a vast number of targets in many cellular compartments, and some display additional enzymatic activities including mono(ADP-ribosyl)ation. SIRTs interact with multiple signalling proteins, transcription factors and enzymes including p53, FOXOs (forkhead box subgroup O), PPARs (peroxisome proliferator-activated receptors), NF-κB, and DNA-PK (DNA-dependent protein kinase). Sirtuins also interact extensively with the family of poly(ADP-ribose) polymerases (PARPs), a crucial and widespread class of NAD^+-consuming post-translational protein modifiers. PARPs share a significant number of roles with sirtuins: these enzymes modulate DNA repair, gene expression, and the activities of signalling pathways.

We focus on the expanding cross-talk between sirtuins, transcription factors and PARPs, which is a highly promising therapeutic target in a number of age-related neurodegenerative disorders, including the most devastating: Alzheimer's and Parkinson's diseases.

Key words: *sirtuins, poly(ADP-ribose) polymerases, FOXO, neurodegeneration, Alzheimer's disease, Parkinson's disease, oxidative stress.*

Introduction

Sirtuins belong to the broad category of histone deacetylases (HDACs), enzymes that modulate signalling proteins, enzymes and transcription factors (TFs) via removal of lysine acetylation. Acylations (including Lys acetylation) are an increasingly recognized, evolutionarily conserved category of post-translational protein modifications; the action of HDACs thus allows highly controlled spatiotemporal regulation of protein activity, interactions and localization. Crucial aspects of cellular homoeostasis depend on acylations including the prevention and mitigation of stress and the removal of the resulting damage. There are over 45 HDAC enzymes identified in eukaryotes, divided into 4 groups (classes) according to their homology to yeast HDACs [38]. Class I enzymes (HDAC1 to -3 and HDAC8) show the stron-

Communicating author

Henryk Jęśko, Department of Cellular Signalling, Mossakowski Medical Research Centre Polish Academy of Sciences, 5 Pawinskiego St., PL-02106 Warsaw, Poland, e-mail: havjes@gmail.com

gest similarity to yeast Rpd3 (reduced potassium dependency 3), while class II enzymes are related to yeast HDA1 and fall into two sub-classes according to the same structural criterion: IIa (HDAC4 to -7 and -9) and IIb (HDAC6, -10). The seven known mammalian class III enzymes are termed sirtuins (SIRT1 to -7; the name stems from a yeast homologue dubbed silent information regulator 2) (Table I). Sirtuins are the only HDACs to use NAD^+ for the reaction; these enzymes localize to various cellular compartments (Table I) [132,207,234] including cytosol (SIRT1, -2), mitochondria (SIRT3-5), and nucleus (SIRT1, -6 and -7, plus cell cycle-dependent transient re-location of SIRT2). Class IV includes only one enzyme, HDAC11.

The unique dependence on NAD^+ availability makes sirtuins excellent sensors of metabolic condition of the cell. Sirtuins transfer the acetyl group removed from a protein to the ADP-ribose moiety of NAD^+; this causes the NAD^+ molecule to break down to nicotinamide and O-acetyl-ADP-ribose (OAADPR), which are SIRT auto-inhibitory compounds. Moreover, OAADPR undergoes rather extensive metabolism and may serve as a signalling molecule capable of modulating gene silencing, ion channel opening,

Table I. Mammalian sirtuin sub-cellular localisation and activities. According to [20,234], modified

	Predicted MW	Primary subcell. localization	Activity	Key targets
SIRT1	80.41; 76.0 kDa[1] 	Nucleus	Deacetylase	p53, FOXO1, 3 & 4, PARP-1; APE1; DNA-PK; RARβ, PGC1α, PPARγ, NFκB, IGF1, histone H1, H3, H4
SIRT2	43.2; 39.5 kDa[2] 	Cytoplasm	Deacetylase	Histone H4, α-tubulin
SIRT3	28.8 kDa; 36.6 kDa[3] ; 43.6 kDa[14] 	Mitochondria	Deacetylase, ADP-ribosyltransferase	Acetyl-coA synthetase, glutamate dehydrogenase, Ku70, isocitrate dehydrogenase
SIRT4	35kDa[15] to 47.3 kDa[4] 	Mitochondria	ADP-ribosyltransferase	Glutamate dehydrogenase
SIRT5	33.8 kDa[5] 	Mitochondria, cytosol[11]	Deacetylase, demalonylase, desuccinylase[10]	Cytochrome c; carbamoyl phosphate synthetase 1; urate oxidase
SIRT6	39.1 kDa[6] 	Nucleus[12], synaptosomes[13]	Deacetylase, ADP-ribosyltransferase	Histone H3; PARP-1; DNA-PK
SIRT7	44.9 kDa[7] 	Nucleus	Deacetylase[9]	RNA Pol I complex; RNA Pol II complex; histone H3[9]; chromatin remodelling proteins[8]

[1]*Mouse; two alternative splicing variants predicted* ***in silico****; Measured MW ~120 kDa [230].*
[2]*Human; two alternative splicing variants predicted [231].*
[3]*Mouse; two alternative splicing variants predicted [232].*
[4]*Mouse [233].*
[5]*Human [234]; http://www.uniprot.org/uniprot/Q9NXA8#Q9NXA8*
[6]*Human [235].*
[7]*Human [236].*
[8]*[237].*
[9]*[238].*
[10]*[6].*
[11]*[239].*
[12]*[7].*
[13]*[240].*
[14]*[241].*
[15]*[242].*

and the function of macro-domain histone proteins [197]. Nicotinamide in turn is also used to re-synthesize NAD^+, and this aspect has additional importance for SIRT activity. However, despite the significant sequence homology between sirtuins, not all of them are deacetylases, and some display other enzymatic activities (Table I). SIRT5 has been found to remove succinyl and malonyl groups from lysines in proteins [47]. SIRT3 and SIRT6 can ADP-ribosylate proteins [113,182] in addition to their deacetylase function [53,90]. Moreover, SIRT4 displays protein mono(ADP-ribosyl)transferase activity and no detectable deacetylation capability [5,71].

Despite the somewhat misleading 'histone deacetylase' term, sirtuins also (un)modify a vast spectrum of non-histone proteins. The targets of SIRT1, which is by far the best characterized sirtuin, include histones, a broad range of stress signalling proteins, and transcription factors (TFs) (Table I). SIRT1 is mainly involved in the regulation of the stress response and macromolecular repair (through its influence on p53 [64], heat shock factor HSF1 [114], forkhead box subgroup O – FOXO proteins [25], peroxisome proliferator-activated receptor – PPAR family [159], Ku70 [85]), anti-inflammatory response (via NF-κB [136,230]), exerts a pro-survival influence (through IIS – insulin/IGF-I signalling [210]), and modulates the generation of mitochondria [66]. Long-term experimental SIRT1 activation *in vivo* is able to retard the onset of age-related metabolic stress and mortality [136]. Its roles in neuronal plasticity/learning and memory phenomena have also been demonstrated [59].

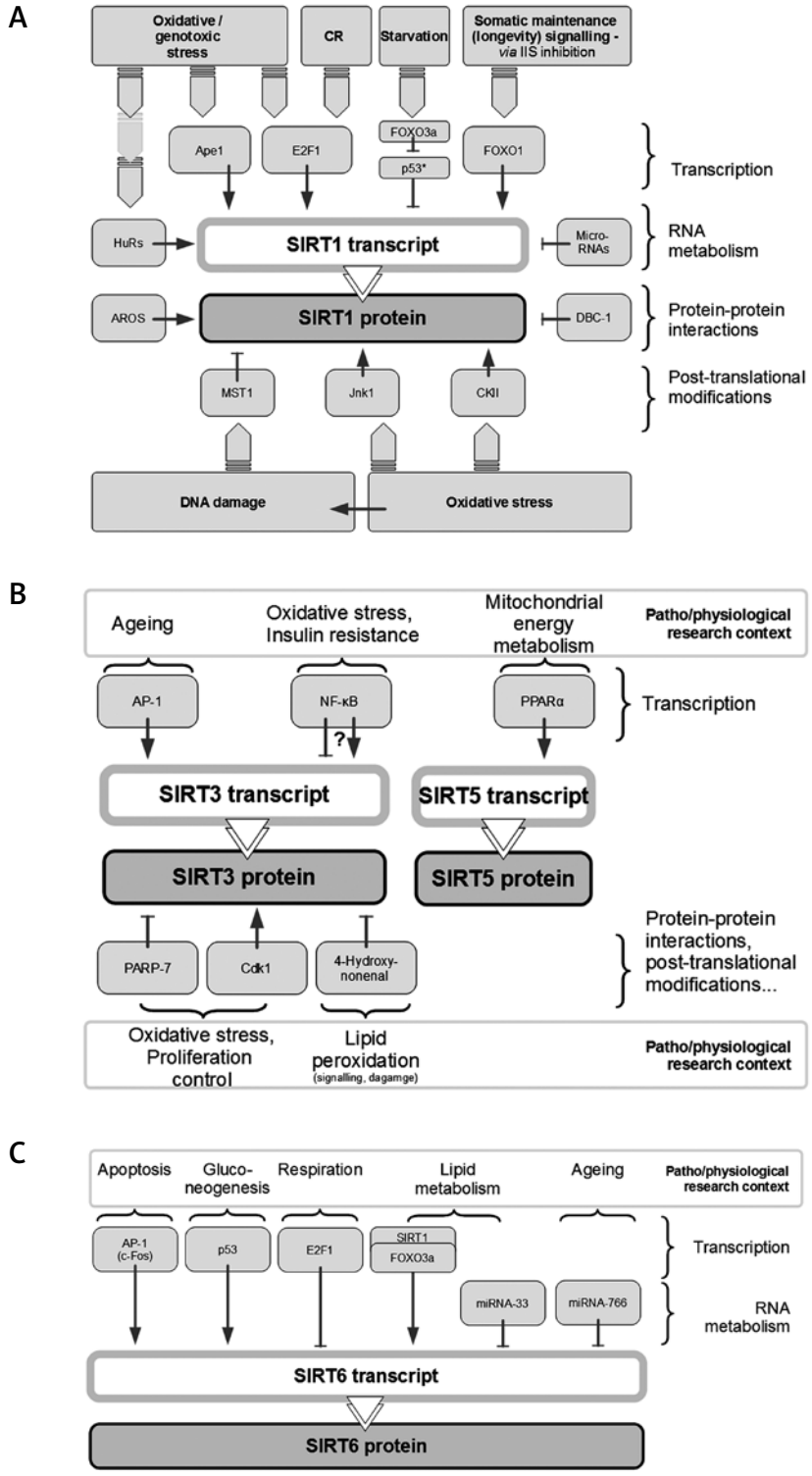
The extensive links of sirtuins with stress signalling, cellular metabolism rates and energy status parallel their cross-talk with the family of poly(ADP-ribose) polymerases (PARPs). PARP-1, the oldest known and best described member of the family, is a 113 kDa protein (in humans) involved in the regulation of chromatin structure, DNA repair, gene expression, and cell death. Its moderate activation is necessary for cellular survival under stress [60]. However, PARP-1 overactivation by glutamate-evoked NO (nitric oxide) production mediates neuronal death in a number of pathological conditions [2,43,188]. The complexity of the enzyme's engagement in the modulation of the cell survival/death equilibrium is additionally reflected by the large changes of its stress response capacity with age [189]. Moreover, the activity of PARPs can be influenced by glutamatergic, cholinergic and possibly other neurotransmission systems [3,63,142], although the significance of this dependency is not fully understood.

An array of interactions has been identified between PARPs and sirtuins, adding to the multiple already described levels of sirtuin regulation (Fig. 1), with increasingly recognized significance for the stress response, metabolic regulation and survival/death decisions.

The multiple levels of sirtuin regulation

SIRT1 to -7 are expressed in the brain and undergo regulation in response to a number of stimuli leading to high regional and developmental variation [169,209] which is modified in the course of ageing [23] and numerous diseases. The transcriptional and post-transcriptional regulation of sirtuins (Fig. 1A-C) occurs at all levels from mRNA expression to post-translational modifications and protein-protein binding.

– A reciprocal relationship links sirtuins with TFs from the FOXO family. Although the majority of findings point to the influence of sirtuin on FOXOs (described below), there are results indicating that FOXOs are able to modulate sirtuin signalling (Fig. 1A, C). The SIRT-1 gene contains several functional FOXO-responsive elements [219]. The signalling between sirtuins and FOXOs extensively cross-talks with the p53 pathway (Fig. 1A, C, Fig. 3A, B). The SIRT1 promoter contains p53-binding sites; p53 interacts there with FOXO3a, mediating the induction of SIRT1 expression by caloric restriction (CR) [144]. In the regulation of glucose metabolism, SIRT6 is important for the p53-dependent nuclear sequestration of FOXO1 [235]. p53 potentially could also impact sirtuins through its links with microRNAs, especially with the *miR-34* family [171].
– A feedback mechanism links SIRT1 with the activity of E2F1 (E2 promoter binding factor), which senses stress conditions (oxidative/stress, CR) [206]: E2F1 activates SIRT1 gene transcription, while SIRT1 exerts feedback inhibition on its TF activity. E2F1 also suppresses *Sirt6* expression, relieving the sirtuin's negative influence on glycolysis in cancer cells [217].
– Oxidative stress activates SIRT1 expression via APE1 (apurinic/apyrimidinic endonuclease-1), a DNA repair endonuclease that possesses much less understood secondary activity as a gene expression regulator [7].



AP-1 – activator protein-1, APE1 – apurinic/apyrimidinic endonuclease-1, AROS – active regulator of SIRT1, CKII – casein kinase II, CR – caloric restriction, Cdk1 – cyclin-dependent kinase 1, DBC-1 – deleted in breast cancer-1, E2F1 – E2 promoter binding factor 1, FOXO – forkhead box subgroup O, HuRs – Hu RNA-binding proteins, Jnk – Jun N-terminal kinase, MST1 – mammalian sterile 20-like kinase 1, NF-κB – nuclear factor κB, PPAR – peroxisome proliferator-activated receptor

**Only selected aspects of p53-dependent modulation are shown; p53 binds a number of sites in the SIRT1 gene, with varying influence on its RNA synthesis and splicing.*

Fig. 1. The multiple levels of sirtuin regulation. **A**) SIRT1, **B**) SIRT3, SIRT5, **C**) SIRT6.

Apart from transcriptional regulation, sirtuin expression has been described to undergo modulation by RNA-binding proteins and non-coding regulatory RNAs.

– The stress-modulated HuR proteins (Hu antigen R, the name derived from the role in the paraneoplastic neurological Hu syndrome) stabilise *Sirt1* mRNA [30] and can influence its alternative splicing [238].
– *Sirt1* mRNA is down-regulated by an antisense long non-coding RNA [214].
– A number of microRNAs also reduce *Sirt1* expression [175], notably in the context of metabolic disturbances, i.e. in the course of obesity-induced changes in fat storage, regulation of mitochondrial numbers, and oxidative energetic metabolism [57]. Persistent down-regulation of *Sirt1* is also observed in ageing. Like in obesity [57], it is caused by elevated *miRNA-34a* [106,184], a proposed brain ageing marker [108] which is capable of modulating cellular senescence [9,83]. A similar effect on senescence has been noted for other microRNAs that target *Sirt1*: *miRNA-22* [81,241] and *miRNA-217* [129]. *Sirt-1* reduction by up-regulated miRNA (*miR-181*) also occurs in the hippocampus of a mouse AD model (3×Tg) [170]. Sirtuin regulation by microRNAs might be in fact a widespread phenomenon in inflammatory and thus possibly neurodegenerative conditions: links exist between *miRNA-34a*, *-132*, *-138*, *-217*, *-373*- and *-520c*-mediated *Sirt1* reduction with NF-κB signalling (at least in the periphery) [49,115,191,220,231], and a reciprocal impact of NF-κB on *Sirt1* expression via miRNA has been noted [94]. Some of the *Sirt1*-regulating miRNAs also respond to oxidative stress, further supporting their potential involvement in neurodegenerative insults [34].

  Apart from Sirt1, also Sirt6 undergoes regulation by microRNAs. Although the results are much less numerous, they also suggest links with aging/senescence and metabolic regulation [37]. Notably, potential feedback regulation between Sirt6 and miRNA-766 modifies the former's role in aging. SIRT6 undergoes reduction by miR-766; with increasing donor age, the re-programming potential of human fibroblasts and the SIRT6 levels fall while miRNA-766 increases. The SIRT6 3'-untranslated region binds miRNA-766 and the microRNA reduces both SIRT6 expression and fibroblast re-programming potential. In turn, SIRT6 reduction could be linked to the increased acetylation of histones observed during ageing in the gene coding for miR-766 [180]. Besides direct suppression, microRNAs can also impact sirtuins indirectly via down-regulation of NAD+ biosynthesis [36], or by affecting IIS components [89], and can mediate IIS' modulation of sirtuins [176].

Beyond the translational level SIRT1 protein binds AROS (active regulator of SIRT1), a protein capable of differentiating its impact upon sirtuin activity depending on the cell status. In response to genotoxic insults in cancer cells AROS supports the inhibitory influence of SIRT1 on p53 [99], while in normal cells the interaction is weak and incapable of modifying SIRT1 activity [102]. SIRT1 also interacts with DBC-1 (deleted in breast cancer-1), which inhibits its enzymatic activity and anti-apoptotic influence, also in a manner dependent on cell phenotype (normal vs. transformed) [10].

SIRT1 protein also undergoes a number of covalent modifications.

– Its nuclear translocation and activation in conditions of oxidative stress is mediated by JNK1 (Jun N-terminal kinase 1)-catalysed phosphorylation [143]. Inhibition of DNA damage-induced, p53-dependent apoptosis by SIRT1 occurs after its phosphorylation by CKII (casein kinase II) [93]. The pro-survival SIRT1 activation also takes place in response to its phosphorylation by DYRK1 and DYRK3 (dual specificity tyrosine phosphorylation regulated kinases) [68]. However, DNA damage may also lead to SIRT1 inhibition, which is done by MST1 (mammalian sterile 20-like kinase 1) [232].
– Lysine SUMOylation (small ubiquitin-like modifier) is an activating event important for SIRT1 activity towards p53; de-SUMOylation of SIRT1 overrides its anti-apoptotic activity in stress conditions [227].
– Activating S-glutathionylation of SIRT1 by the redox-modulated enzyme glutaredoxin 2 may be critical to the sirtuin's role in vascular development [24].

Besides these specific mechanisms of regulation, the activity of sirtuins has also been shown to be post-translationally de-stabilized and inhibited by products of oxidative damage to lipids such as 4-hydroxynonenal [27,56].

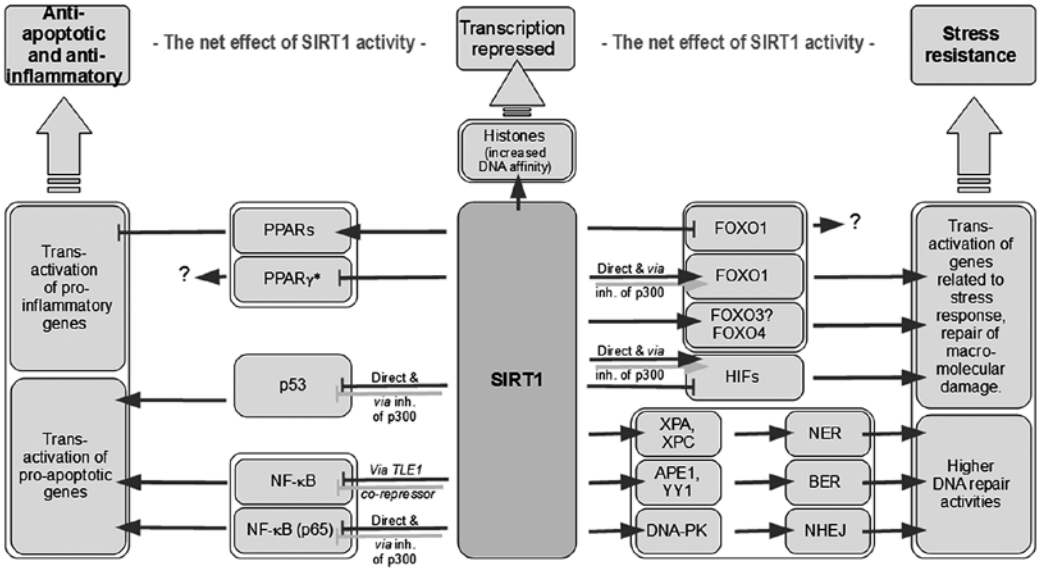
A number of further protein-protein interactions and post-translational sirtuin modifications are described below. They form part of the multiple feedback regulatory loops connecting sirtuins with their signalling targets.

Transcriptional and post-transcriptional regulators as sirtuin targets

Sirtuins post-translationally regulate vast numbers of proteins including histones, TFs and co-activators, and enzymes (Table I, Fig. 2). Deacetylation restores the affinity of inactivated core histones to DNA, thus allowing general gene silencing [82] (Fig. 2). This mechanisms may constitute one of the ways sirtuins reduce overall metabolic rates [145] and improve neuron survival. However, binding to specific promoters (e.g. via interactions with sequence-specific proteins there) allows sirtuins to modify histones and affect chromatin structure also in a localized manner [21].

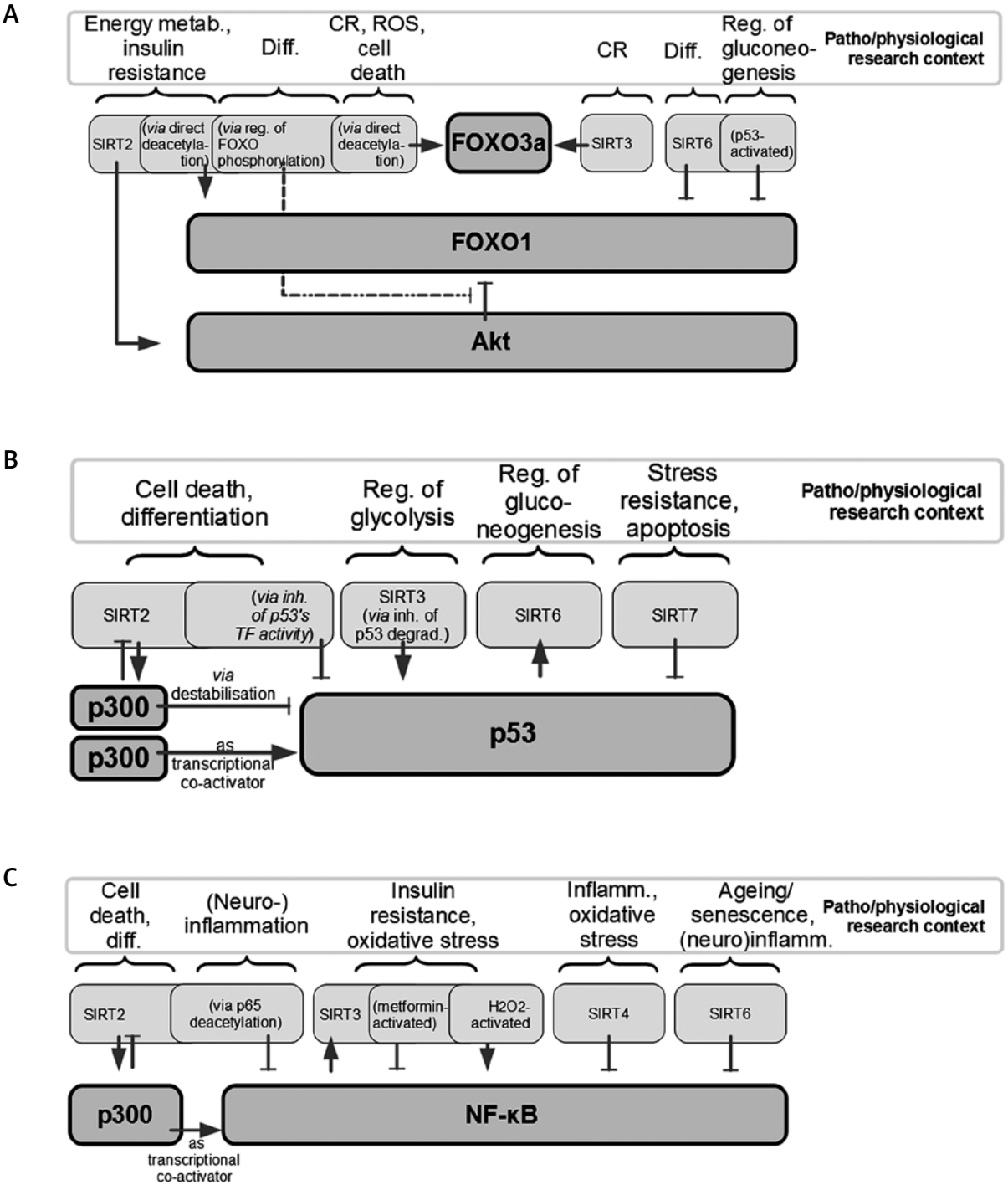
Interactions with transcription factors is a major mechanism of sirtuins' influence on metabolism and cell fate. The links between TFs of the FOXO family and sirtuins are extensive (Figs. 2 and 3) [219]. Sirtuins modulate FOXOs directly; moreover, sirtuins also add another level of FOXO regulation via modulation of the IIS pathway:

– SIRT1 deacetylates FOXO1 (Fig. 2) with varying effects on its activity: FOXO1 deacetylation increases its TF activity on SIRT1 and some other genes [219] while suppressing it in other situations (possibly due to different protein complex composition/promoter sequence) [228]. SIRT1 also modulates FOXO1 through enhancement of its nuclear presence [55] and probably changes its target gene spectrum [62]. SIRT2 in turn facilitates DNA binding by FOXOs [207]; deacetylation by SIRT2 inhibits the Akt-mediated nuclear sequestration of FOXO1 [88] (Fig. 3C). This enhances the inhibitory influence FOXO1 exerts on PPARγ, thus mediating the changes in adipose metabolism induced by nutrient deprivation or exposure to low temperature [208]. FOXO3 and FOXO4 are also deacetylated by SIRT1 and 2; this exerts a complex influence on their downstream mediators including superoxide dismutase, $p27^{kip1}$, and GADD45 (growth arrest and DNA damage 45) and target processes such as stress resistance cell cycle and death [25,78,101,173,207].
SIRT3 is a necessary partner in the mitochondrial gene expression control by FOXO3a. CR (caloric restriction) causes FOXO3a to accumulate in mitochondria, where it interacts with SIRT3 and with RNA polymerase to activate gene expression, which boosts mitochondrial respiration [155].
– FOXOs also are modulated indirectly via insulin(-like) signalling (IIS)/Akt. The outcome varies depending on the different sirtuins involved and cell lines used.



**PPARγ inhibition by SIRT1 exerts a much less clearly understood immunomodulatory role.*

Fig. 2. SIRT1 signalling targets with potential impact on neurodegenerative processes. According to [234], modified. BER, base excision (DNA) repair; DNA-PK, DNA-dependent protein kinase; inh., inhibition; NER, nucleotide excision repair; NHEJ, non-homologous end-joining (DNA repair); XPA, xeroderma pigmentosum group A; XPC, xeroderma pigmentosum group A; YY1, yin yang 1.



CR – caloric restriction, Diff. – cellular differentiation, inh. – inhibition, reg. – regulation, ROS – reactive oxygen species

Fig. 3. Signalling network of SIRT2 to -7. **A**) Interactions of sirtuins with p53 and its co-activator p300. **B**) Sirtuins, NF-κB and its co-activator p300. **C**) FOXO transcription factors and sirtuins.

SIRT1 enhances IIS signalling – it occurs through at least two ways:

- deacetylation of p53 leads to reduction of its protein levels, relieving IIS inhibition by the IGF-binding protein-3 [215];
- SIRT1 can also directly deacetylate Akt, restoring its ability to bind phosphoinositides and become activated by phosphoinositide-dependent protein kinase 1 [192]).

These dependencies have already been confirmed to impact metabolic deregulation, cardiac dysfunctions and tumour formation, and might result in inhibition of the IIS target FOXO1 [67].

In addition to SIRT1, also SIRT2 can physically interact with Akt; the sirtuins may be *exchanged* depending on the activation state of the IIS pathways. SIRT2 is necessary for full activation of Akt in response to insulin/growth factor signalling, while a deficient Akt response is noted in metabolic disturbances including insulin resistance [164]. Together with the above-mentioned results it suggests an image of extremely tightly regulated, multi-level influence of SIRT2 on FOXO-mediated events.

Besides direct interactions with FOXO3a, SIRT3 also has a potential indirect impact on FOXOs by moderating Akt overactivation by ROS [158].

SIRT6 has been shown to suppress IIS signalling-modulated genes [194], resulting in reduced FOXO1 expression [193]. SIRT6 also mediates p53-induced nuclear sequestration of FOXO1 in the regulation of energy metabolism [235].

The FOXOs' extensive interactions with various stress signalling and protein turnover pathways allow them to mediate a broad spectrum of homeostatic responses. Their role in the longevity/neuroprotective effects of IIS (insulin/insulin-like signalling)-dependent modulation of stress resistance is of particular importance. FOXOs' links may be crucial for the pathomechanism of a number of (mostly age-related) diseases associated with disturbed somatic maintenance, including AD, leading to suggestions that they could constitute targetable *integrating factors* influencing various neurodegenerative mechanisms [125].

The highly conserved tumour suppressor p53 and its paralogues (p63, p73) have long been known to take part in the DNA damage response, especially cell cycle arrest, cellular senescence, and death. These TFs are also capable of direct modulation of DNA repair genes and proteins [146]. Moreover, the p53 family could also be linked to ageing at the organism level [146,160]. Other emerging roles of p53 in glucose and lipid metabolism, ROS signalling and oxidative stress [64] suggest a significant functional overlap with SIRT pathways. p53 undergoes extensive post-translational modifications of several types; this makes it sensitive inter alia to inhibition and destabilisation via sirtuin-catalysed deacetylation (Figs. 2 and 3B). Moreover, SIRT1 binds and inhibits p53 promoter [52]; SIRT1's interactions with the senescence modulator *miRNA-34a* also allow a post-transcriptional influence on p53 [77,229], while both SIRT1 and p53 can be miRNA-34a's targets as well [229]. SIRT1 expression increases in the conditions of H_2O_2-induced oxidative stress, and sirtuin activation inhibits p53-dependent apoptosis [240]. Down-regulation of SIRT1's influence on p53 mediates responses to several stressors in other cell types [204,224] and to a range of age/hyperglycaemia-related vascular endothelial pathologies [107,233]. Similar mechanisms of age-related, glucose-elicited damage might also be involved in neurodegenerative disorders along with generalized oxidative/nitrosative stress. Indeed, it is suggested that a significant part of SIRT1's neuroprotective signalling could be mediated through p53 [234], including SIRT1's roles in AD and PD [39,98,151]. Sirtuin-mediated changes in p53 stability and TF activity also occur in an experimental model of hippocampal neuronal plasticity [112].

Less characterised sirtuin family members have also been noted to signal through p53 (Fig. 3B). Administration of a SIRT2 inhibitor resulted in increased p53 acetylation [226]. The influence of SIRT2 on p53 appears to be complex; it can either block its trans-activating influence on gene expression (via direct deacetylation) [87], or enhance its degradation [18], sometimes only when working in concert with SIRT1 [153]. SIRT3 is able to modulate p53 degradation mediated by MDM2 (mouse double minute 2 homolog), and the influence p53's role as a metabolic regulator [237]. SIRT6 also takes part in p53's modulation of energy metabolism via nuclear sequestration of FOXO1 [235]. A recently identified cytoplasmic pool of SIRT7 binds p53 in a complex with TPPII (tripeptidyl-peptidase II, also capable of modulating NF-κB) [141].

Moreover, sirtuins also interact with an important partner of p53 and NF-κB, p300 (Figs. 2 and 3B). p300 is a transcriptional co-activator able to block the interaction of histones with DNA through their acetylation. However, p300 is also able to reduce p53 stability via its negative regulator MDM2, in a manner that appears to depend on the type of upstream signals or on cell type [109]. SIRT1 can inhibit the acetylating activity of p300 [22], which might exert a pro-survival influence in AD [48]. However, the influence of SIRT2 on p300 appears to be opposite to that of SIRT1 [18], as mentioned above in the context of p53 degradation. In turn, p300 inhibits SIRT2 through acetylation, attenuating its negative influence on p53 [73].

The NF-κB pathway has been proposed to be a nearly universal booster of the innate immunity and pro-inflammatory responses that largely counteracts the FOXO system [173]. NF-κB activity often significantly contributes to neuronal damage in AD, ischaemia, and other disorders; the blockage of NF-κB-dependent gene transactivation by sirtuin signalling offers neuroprotection in amyloid β (Aβ) toxicity [32]. Moreover, the regulatory activities of NF-κB are altered during ageing [76], while NF-κB is capable of modulation of ageing/senescence largely via its sirtuin interactions [96]. Despite varying intracellular localisations and interactions repertoires, most sirtuins modulate NF-κB, often in a negative manner.

- SIRT1 inhibits NF-κB (Fig. 2) through:
  - deacetylation of the RelA subunit of NF-κB (this RelA modification is dependent on p300 or PCAF – the p300/CBP-associated factor) [230];
  - interactions with NF-κB's transcriptional co-repressor TLE1 (transducin-like enhancer protein 1) [61].
- SIRT2 is also able to inhibit the TF via deacetylation of p65 (Lys 310) (Fig. 3C); [117]. However, its known positive influence on p300 [18] suggests that the regulatory interactions between these proteins might be significantly more complex than currently known.
- SIRT3, itself a transcriptional target of NF-κB [116], mediates the inhibitory effect of metformin on NF-κB in a cellular model of oxidative stress and insulin resistance [185]. In contrast, in a different cell line SIRT3 has been found to activate H_2O_2-induced, NF-κB-dependent expression of, inter alia, superoxide dismutase [31], strongly suggesting that the interaction is promoter-specific and/or modified by further interactions.
- SIRT4 blocks the degradation of IκB (inhibitor of κB) [33] and reduces the nuclear translocation of NF-κB and resulting pro-oxidative and pro-inflammatory phenotype [196].
- SIRT6 binds RelA and is able to repress NF-κB target promoters that become activated during aging [96], and can delay cellular senescence [218]. However, the effect has not been observed in some other models/conditions [65], possibly due to the dynamic and interdependent character of the interaction with NF-κB [97].
- Besides these, the sirtuin target FoxO3a interacts with NF-κB [111] and with its PI-3K (phosphoinositide 3-kinase)/Akt-dependent upstream activator IKKβ (IκB kinase β) [154], which suggests additional paths of influence.

Sirtuins thus simultaneously impact the pro-inflammatory and potentially deleterious actions of NF-κB and activate FOXO somatic maintenance signalling [173]. The effect may modulate the stress resistance signals of IIS, which is able to regulate both FOXOs and NF-κB [70].

The family of hypoxia-inducible factors (HIFs) modulates, inter alia, energy metabolism and the stress response depending on oxygen concentration. SIRT1 inhibits HIF1 [110] but activates HIF2 (Fig. 2) [45], while SIRT6 may be a co-repressor for HIF-1α [242]. The significance of this discrepancy has not been extensively tested, but invertebrate data suggest engagement of HIFs in the modulation of ageing rates. Moreover, HIFs' transactivation targets include genes with known neuroprotective products, although it has been suggested that these TFs might play either protective or detrimental roles [54,86,140, 223].

The sirtuin interaction partners peroxisome proliferator-activated receptors (PPARα, PPARβ/δ, PPARγ) are a class of nuclear receptors, TFs whose intracellular localization and activity are regulated by ligand binding. PPAR roles include metabolic regulation in response to environmental cues, proliferation control, and cardiovascular homoeostasis; they modulate oxidative stress, inflammation, or insulin resistance. PPARs can antagonize neurodegeneration in AD/PD/cerebral ischaemia/brain trauma [139,161]. They may also be of therapeutic interest in the metabolic syndrome [58]. PPARs also modulate inflammation that partially mediates these pathologies

[58,139,161,165]. PPARs may also constitute plausible targets in diabetes and diabetes-linked neuropathy.

SIRT1 is involved in a two-directional interaction with PPARα. SIRT1 binds PPARα on its DNA response elements. The binding is tightly regulated depending on the DNA sequence [150]. The resulting deacetylation enhances PPARα activity [167] (Fig. 2). SIRT1 also facilitates the protein-protein interaction between PPARα and NF-κB (p65) [159]. *Sirt1* and *PPARα* genes are regulated in a coordinate manner by the ageing-linked *miRNA-22* [69] and *miRNA-34a* [44], while SIRT1 is able to modulate *miRNA-34a* in concert with p53 [77]. This suggests a precisely regulated feedback mechanism, but the potentially significant topic has not been explored much further. The widely used natural sirtuin activator resveratrol has been shown to bind and activate PPARα directly [195]. SIRT1 also reverses the p300-dependent acetylation of PPARγ [72] and seems to inhibit its transactivation function [156]. PPARα, PPARγ, and PPARδ agonists were able to increase SIRT1 expression [35,100,213]; PPARα activation also blocked SIRT1 export from the nucleus [213]. The *Sirt5* gene promoter contains potential PPARα-responsive sequences, and the PPARα agonist is able to increase its expression [26]. Besides SIRT1, also SIRT6 displays links with the signalling network of PPARs [225].

Not surprisingly, the interactions between sirtuin and PPAR pathways profoundly modulate energy metabolism [26] and appear to have an impact on a number of pathophysiological conditions (Fig. 2). SIRT1 is involved in a potential senescence-related feedback interaction with PPARγ [72]. PPARγ is widely present in the brain (neurons and microglia), lowers local levels of iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2), and might constitute an effective target in the treatment of ischaemia [42]. Moreover, the impact of metabolic stress on SIRT1-PPARγ signalling has been suggested to modulate β-secretase and thus the rate of amyloid β production in AD [211]. Additionally, differential expression of *Sirt1* and *PPARγ* has been noted in Aβ-treated glia, which would fit the above-mentioned antagonistic regulation of *Sirt1* by PPARγ; it has been proposed to mediate the neuroprotective reaction of astrocytes elicited by *in vitro* Aβ treatment [4]. Outside the brain, PPARα is one of the effectors of SIRT1's cardioprotective actions [159], although in some circumstances the SIRT1-PPARα interaction may actually promote heart hypertrophy [149].

PPARγ co-activator 1α (PGC-1α) is an important player in the PPAR network, capable of modulating respiration/oxidative stress resistance [183] and neuronal survival. Its ASN-induced [221] disturbances may be implicated in the pathogenesis of Parkinson's disease [40], and PGC-1α has been proposed as a therapeutic target in PD [239].

PGC-1α regulates mitochondrial biogenesis by working together with SIRT1 [8]. SIRT1 reverses the p300-mediated acetylation of PGC-1α in a unique nuclear-mitochondrial cross-talk [8]. Additionally, SIRT1 binds the PGC-1α promoter and takes part in its positive regulation loop [6]. An interesting interaction takes place between PGC-1α and SIRT6: the sirtuin deacetylates and activates the acetyltransferase GCN5 (general control non-repressed protein 5), which leads to increased acetylation of PGC-1α and inhibition of its transcriptional co-activator function [46]. PGC-1 has been proposed to mediate the protective SIRT1/PPAR-dependent action of Aβ-challenged astrocytes towards neurons (the increase of neuronal biogenesis of mitochondria and survival in the co-culture with astroglia) [4].

AP-1 (activator protein-1) is a dimeric TF consisting of proteins from Fos and Jun families, with a wide variety of roles in development, cell proliferation, survival and migration, and ROS (reactive oxygen species)/low oxygen signalling [133,181]. AP-1 has been implicated in the control of brain plasticity and damage [162], including a hypothesized central role in AD/PD [166], and of numerous peripheral functions.

SIRT1 exerts a varied, context-specific influence on the transactivation of genes by AP-1 to modulate processes ranging from cyclooxygenase expression to pathogen replication [168,236]. The *Sirt3* gene contains an AP-1 binding site [15] in its longevity-correlating intronic enhancer [16]. As alleles displaying the lowest activity of this enhancer are notably absent from the oldest old group, the interaction may have strong significance for the modulation of human lifespan [16]. SIRT6 (which has also been associated with lifespan modulation via IIS [92]) binds c-Jun, undergoes recruitment to its target promoters and reduces their activity via histone deacetylation [193]. c-Fos is able to induce transcription of the *Sirt6* gene; the sirtuin in turn represses survivin via NF-κB. The significance of apoptotic resistance regulation

by AP-1–SIRT6 signalling in the survival of pre-neoplastic lesions is further strengthened by the observation that both display specific expression patterns in pathological tissue samples [134].

Further elucidation should cast more light on sirtuin–AP-1 cross-talk, which could have significant consequences for, inter alia, brain development, homeostasis, learning and memory, and neurodegenerative conditions [162].

While microRNAs are an emerging mechanism of sirtuins' gene regulation, relatively little is known about the possible specific impact of sirtuins on miRNA metabolism (see above, PPAR section).

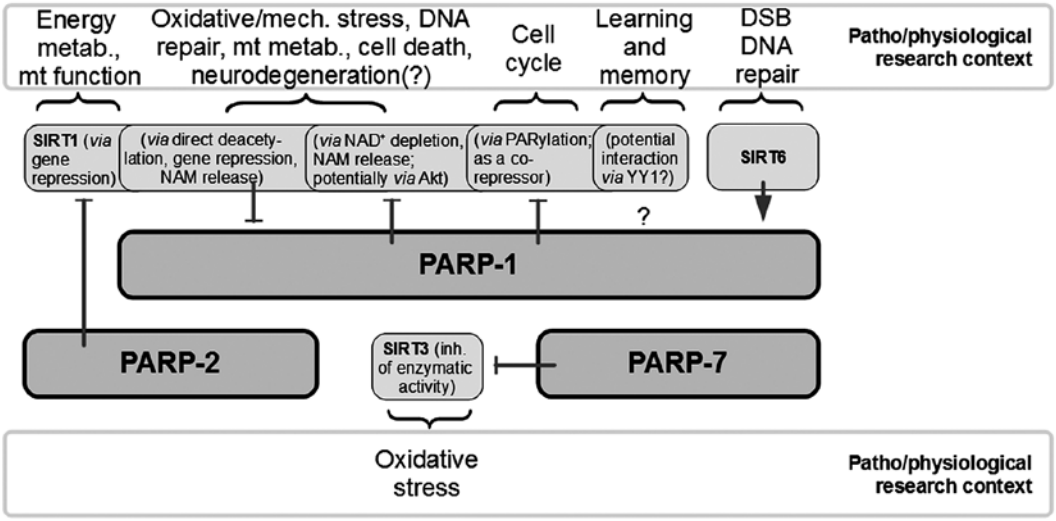
Sirtuins and DNA repair

The interaction with stress-related TFs may have vast significance for the regulation of DNA repair by sirtuins. Additionally, some TFs (such as FOXO3a, p53, NF-κB, E2F1, Sp1 – specificity protein-1, or some nuclear receptors) have also been implicated in the repair process itself, possibly via relaxation of chromatin structure, though the matter still needs some clarification [124,201]. However, sirtuins are able to directly influence proteins involved in the repair of macromolecular damage.

– Apurinic/apyrimidinic endonuclease 1 (APE1) is one of the crucial factors involved in the base excision repair (BER) pathway which removes the ubiquitous products of free radical-related damage from DNA. APE1 has been shown to be inactivated by acetylation at multiple sites [222]. SIRT1 binds APE1 and deacetylates two of its lysines. This stimulates APE1 to bind its partner XRCC1 (X-ray cross-complementing-1) and increases its activity in the BER complex. The net effect of sirtuin-mediated stimulation of APE1 is an improvement of the efficiency of this crucial repair mechanism, as measured by reduced levels of abasic sites in DNA [222].
– SIRT1 is also known to facilitate the activity of nucleotide excision repair (NER), a mechanism that removes a wide spectrum of DNA lesions/adducts and has demonstrated crucial significance in cancer prevention. SIRT1 deacetylates two lysines of the core NER protein XPA (xeroderma pigmentosum group A); this reaction is necessary for the full efficiency of UV damage removal [50]. SIRT1 also relieves the repression of the *XPC* gene coding for a protein that recognizes DNA lesion and recruits other NER components [135].

Table II. Mammalian PARP enzymes. According to [79,205], modified

Old name	New unified name	Activity – measured	Activity – postulated
PARP1	ARTD1	PARylation	
PARP2	ARTD2	PARylation	
PARP3	ARTD3	Mono(ADP-ribosyl)ation	PARylation
vPARP/PARP4	ARTD4	Mono(ADP-ribosyl)ation	PARylation
Tankyrase-1/PARP5a	ARTD5	PARylation	
Tankyrase-2/PARP5b/PARP6	ARTD6	PARylation	
PARP6	ARTD17	Mono(ADP-ribosyl)ation	
PARP7	ARTD14	Mono(ADP-ribosyl)ation	
PARP8	ARTD16	Mono(ADP-ribosyl)ation	
PARP9	ARTD9	Not detected	
PARP10	ARTD10	Mono(ADP-ribosyl)ation	
PARP11	ARTD11	Mono(ADP-ribosyl)ation	
PARP12	ARTD12	Mono(ADP-ribosyl)ation	
PARP13	ARTD13	Not detected	Mono(ADP-ribosyl)ation (mouse)
PARP14	ARTD8	Mono(ADP-ribosyl)ation	
PARP15	ARTD7	Mono(ADP-ribosyl)ation	
PARP16	ARTD15	Mono(ADP-ribosyl)ation	



Mt metab. – mitochondrial metabolic regulation, mech. stress – mechanical stress, NAM – nicotinamide

Fig. 4. Interactions between sirtuins and poly(ADP-ribose) polymerases.

– DNA-dependent protein kinase (DNA-PK) is involved in non-homologous end-joining (NHEJ) repair, which neutralises double-strand breaks, a highly mutagenic and lethal type of DNA lesion. DNA-PK is also an anti-apoptotic signalling protein. The Ku70 subunit of DNA-PK undergoes inhibitory acetylation on at least 8 lysines by, inter alia, PCAF (p300/CBP-associated factor), a histone acetyltransferase that also collaborates in DNA damage signalling with p53 [179]. SIRT1 associates with and deacetylates Ku70, thus activating DNA-PK in both its roles [41,85]. SIRT6 also appears to be involved in DNA maintenance [21] and modulates the binding of DNA-PK to regions of DNA double-strand breaks, thus facilitating the removal of these deleterious lesions [127].
– SIRT6 was observed to be an important factor in telomere maintenance through deacetylation of histone H3. Moreover, SIRT6 appears to stabilize the association of Werner protein with telomeric chromatin, further contributing to the regulation of its architecture [131].

Sirtuins and PARPs

Sirtuins interact in a complex way with the versatile family of Poly(ADP-ribose) polymerases (PARPs) (Table II, Fig. 4). The roles of various PARPs include DNA repair (modulated chiefly by PARP-1 to -3), regulation of gene transcription (PARP-1, -2, and structurally different *macroPARPs*: PARP-9, -14, -15) [128], RNA processing in the nucleus and cytoplasm (PARP-1, -7, -10, -12 to -15, tankyrase-1) [19], cellular RNA transport (probable role of vault PARP and PARP-10) [1], cellular transport of proteins (mainly PARP-16) [1], and telomere maintenance (somewhat ambiguously including PARP-1, tankyrase-1 and possibly tankyrase-2) [174,177].

The family's founding member PARP-1 detects DNA damage (single- and double-strand breaks, abnormal spatial structures) and post-translationally modifies histones to locally de-condensate chromatin, thus facilitating access for the repair machinery [190]. It also directly recruits and modulates DNA repair proteins involved in BER, NER, NHEJ, and homologous recombination DNA repair pathways, and numerous signalling proteins [188]. Besides regulating chromatin accessibility [199], PARPs can act more specifically, as activators/co-activators or (co-) repressors for numerous TFs. PARP-1 modulation of transcription factors impacts both gene regulation and the recently identified role of TFs in DNA repair [84,124].

The extensive network of interactions between PARP-1 and the p53 pathway cross-talks with other post-translational modifiers [216], possibly including sirtuins [138], with vast significance for most previously identified PARP functions [74]. The co-operation between PARP-1 and numerous TFs also includes NF-κB and is important for neurodegeneration in Alzheimer's disease [95], for brain ischaemia [80], etc.

Despite the pro-survival physiological significance of PARP-1, its excessive activation by DNA damage induced by ROS/RNS (reactive nitrogen species) [187], Aβ, or mutagens [189] has long been associated with cell death. The long-postulated theory of passive cellular demise via stress-induced energy imbalance suggested that PARP over-activation by intense DNA damage would lead to massive PARylation, depleting cellular stores of NAD^+ and consequently ATP (which is used to re-synthesize it). However, more recent works have suggested that in post-mitotic cells nuclear NAD^+ depletion itself could be more significant, inhibiting some crucial enzymes that utilise the nucleotide as a substrate [157]. PARP-1's K_M towards NAD^+ should be low enough to make it relatively insensitive to the changes of NAD^+ concentration and to allow continued activation despite ongoing metabolic disruption. In contrast, the nuclear SIRT1 displays K_M closer to the reported intracellular NAD^+ levels and thus should be significantly influenced by such pathophysiological changes [28,157]. Indeed, cell death caused by PARP over-activation was rescued by various interventions that boosted NAD^+ levels and occurred only in the presence of the intact SIRT1 orthologue Sir2α [157]. Increased activity of SIRT1 in PARP-$1^{-/-}$ mice was also noted [12]. The PARP–sirtuin substrate competition has already been confirmed to impact SIRT1 downstream events linked to the regulation of cell death/survival [157] or mitochondrial metabolism [12]. Disruption of the SIRT1-PGC-1α axis by (over)activated PARP-1 has been suggested to be of significance for the pathomechanism of several DNA repair disorders accompanied by neurodegeneration where mitochondrial abnormalities may play significant roles [51,178,200]. SIRT1 inhibition via NAD^+ depletion might also mediate other neurodegenerative insults such as the death of hippocampal cells in culture in a model of acute epileptic neuron loss [212].

Sirtuins other than SIRT1 also display K_M that would suggest dependency on PARP-induced NAD^+ fluctuations. However, the phenomenon of inactivation by PARP-mediated substrate competition appears to be restricted to SIRT1. The (in)sensitivity of various sirtuins to competition with PARP-1 might stem from several factors, including their intracellular localisation and their ability or not to pre-bind NAD^+ and thus escape the NAD^+ depletion [28]. Moreover, in some situations sirtuin inhibition by oxidative stress may be direct and not mediated by the competition with PARPs for the substrate [27].

Yet other mechanisms of cross-talk might exist, as both PARP-1 [147,148] and SIRT1 [17] interact with YY1 (yin yang 1). YY1 is an important regulator of miRNAs and protein-coding genes related to neuronal plasticity [59] and degeneration [104] as well as DNA repair [147]. A potentially significant topic for sirtuin regulation is the observed impact of PARP-1 on both upstream modulators and signalling targets of sirtuins. PARP-1 appears to be critically involved in the modulation of Akt activity [91,186]; however, despite its importance for, inter alia, neurodegeneration [130], or ischemic damage [105] the mechanism of this interaction has not been explored further. PARP-1 also directly binds and PARylates FOXO1, leading to suppression of FOXO1-dependent genes [172].

The more favourable K_M of PARP-1 should allow it to *out-perform* SIRT1 in the competition for NAD^+ in all situations [12]. However, both enzymes are able to block each other's activity by releasing the inhibitory by-product nicotinamide [103]. SIRT1 has also been able to mitigate the rapid PARP-1 activation in oxidative (H_2O_2-induced) stress while SIRT1 knock-out has led to enhanced apoptotic signalling and cell loss in these conditions [103]. The results obtained by Rajamohan suggest that depending on the conditions the difference in K_M could be negligible: the value for PARP-1 activated by pERK or the histone acetyltransferase PCAF (p300/CBP-associated factor) is just 10% to 20% lower than that of SIRT1 [163].

The activation of PARP-1 by PCAF in stress conditions occurs via acetylation [163], making it a good substrate for SIRT1. SIRT1 has been shown to interact with and de-acetylate PARP-1 [163], reversing its enzymatic stimulation and reducing it to nearly undetectable levels. Surprisingly, acetylation boosted only the basal activity of PARP-1 and not its maximum, DNA damage-induced activity. However, removal of this modification inhibited PARP's (mechanical stress-related) activation, thus potentially offering some cytoprotective potential [163]. Although the physical interaction between SIRT1 and PARP 1 is dependent on NAD^+ availability and gradually diminishes with its increasing concentration, SIRT1 pre-bound to NAD^+ is still able to bind PARP-1 physically (and possibly deactivate it) despite the lack of substrate. This suggests a potential mechanism for pre-

serving SIRT1 activity despite NAD^+ depletion [163]. Most work on SIRT1-mediated PARP-1 inhibition has been done on cell lines of non-neuronal origin. However, it has been shown that the influence of SIRT1 on PARP-1 can indeed be of significance in oxidative stress conditions, thus raising hopes for using it as a potential target in neurodegeneration. The absence of SIRT1 sensitized the cells via PARP to H_2O_2-induced death [103], while over-expression of SIRT1 in HeLa cells reduced PARP-mediated, DNA damage-induced death in a mode dependent on its deacetylase function [163].

SIRT1 is capable of modulating not only PARP-1 protein but also its gene expression. SIRT1 over-expression in cardiomyocytes has been shown to reduce *PARP-1* gene promoter activity and *PARP-1* mRNA, which translated into lower protein levels; deacetylase activity was necessary for the effect. SIRT1 did not appear to influence the degradation of PARP-1 protein, as shown in experiments with proteasomal and lysosomal inhibitors [163].

Other PARPs (Figs. 1B and 4; Table II), whose activities typically fall well below those of PARP-1, are able to modulate sirtuins in ways independent of NAD^+ fluctuations. [11]. PARP-2 is a direct negative regulator of the SIRT1 promoter, and its impact on the SIRT1 gene has direct consequences for energetic metabolism and mitochondrial function (Fig. 4) [137]. PARP-7, or tetrachlorodibenzo-p-dioxin-inducible poly(ADP-ribose) polymerase (TiPARP), appears to have the ability to inhibit SIRT3 activity (but not mRNA expression) in conditions of oxidative stress (Fig. 4); this leads to reduced expression of superoxide dismutase-2 and might further exacerbate the damage [75].

It is not clear if the acetylated residues present in PARPs other than PARP-1 could be targeted by sirtuins or if these isoforms are able to significantly affect SIRT activities.

The influence of SIRT2 to -7 on PARPs is not fully determined. A rather unusual interaction takes place between SIRT6 and PARP-1 [126]. SIRT6 resides largely in the heterochromatin; it is recruited to double-strand break sites and its expression is enhanced in response to DNA damage. Its stimulatory effect on DNA repair was visible both under resting and stress conditions evoked by paraquat (producing superoxide), neocarzinostatin (a single- and double-strand break inducer) or H_2O_2. SIRT6 physically binds PARP-1 in a manner enhanced by the damage and mono(ADP ribosyl)ates it on Lys521. PARP-1 enzymatic activity is stimulated by this interaction and mediates the positive effect of SIRT6 on the efficiency of NHEJ and homologous recombination repair (Fig. 4); [126]. Although SIRT6 did not influence the acetylation level of PARP1, both SIRT6 enzymatic activities have been found to take part in the regulation of DNA repair [126]. The opposite influence of SIRT1 and -6 on PARP activity prompted Cantó et al. to suggest that these proteins could constitute a signalling switch in the DNA repair network [28]. In an example scenario, ATM (ataxia-telangiectasia mutated), which senses DNA damage, would phosphorylate DBC-1 protein, facilitating its inhibitory influence on SIRT1. This would remove the inhibition of PARP-1, thus leaving only the positive influence of SIRT6 and allowing PARP-1 to efficiently perform its protective function [28].

The described unique characteristics of sirtuins correspond to their broad links to signalling pathways and enzymes involved in cellular maintenance and the stress/damage response. Some sirtuins localise to mitochondria and modulate their biogenesis as well as the function of the respiratory machinery. Moreover, sirtuins are capable of influencing anti-oxidative proteins and the unfolded protein response there, as well as the mitochondrial cell death signalling. A growing body of evidence links sirtuins to aging and neurodegenerative diseases, making these HDACs highly promising research and therapeutic targets.

Acknowledgments

The authors' work is supported by National Science Centre grant 2013/09/B/NZ3/01350.

Disclosure

Authors report no conflict of interest.

References

1. Abd Elmageed ZY, Naura AS, Errami Y, Zerfaoui M. The poly(ADP-ribose) polymerases (PARPs): new roles in intracellular transport. Cell Signal 2012; 24: 1-8.
2. Adamczyk A, Czapski GA, Jeśko H, Strosznajder RP. Non A beta component of Alzheimer's disease amyloid and amyloid beta peptides evoked poly(ADP-ribose) polymerase-dependent release of apoptosis-inducing factor from rat brain mitochondria. J Physiol Pharmacol 2005; 56 Suppl 2: 5-13.
3. Adamczyk A, Jeśko H, Strosznajder RP. Alzheimer's disease related peptides affected cholinergic receptor mediated

poly(ADP-ribose) polymerase activity in the hippocampus. Folia Neuropathol 2005; 43: 139-142.
4. Aguirre-Rueda D, Guerra-Ojeda S, Aldasoro M, Iradi A, Obrador E, Ortega A, Mauricio MD, Vila JM, Valles SL. Astrocytes protect neurons from Aβ1-42 peptide-induced neurotoxicity increasing TFAM and PGC-1 and decreasing PPAR-γ and SIRT-1. Int J Med Sci 2015; 12: 48-56.
5. Ahuja N, Schwer B, Carobbio S, Waltregny D, North BJ, Castronovo V, Maechler P, Verdin E. Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. J Biol Chem 2007; 282: 33583-33592.
6. Amat R, Planavila A, Chen SL, Iglesias R, Giralt M, Villarroya F. SIRT1 controls the transcription of the peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) gene in skeletal muscle through the PGC-1α autoregulatory loop and interaction with MyoD. J Biol Chem 2009; 284: 21872-21880.
7. Antoniali G, Lirussi L, D'Ambrosio C, Dal Piaz F, Vascotto C, Casarano E, Marasco D, Scaloni A, Fogolari F, Tell G. SIRT1 gene expression upon genotoxic damage is regulated by APE1 through nCaRE-promoter elements. Mol Biol Cell 2014; 25: 532-547.
8. Aquilano K, Vigilanza P, Baldelli S, Pagliei B, Rotilio G, Ciriolo MR. Peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and sirtuin 1 (SIRT1) reside in mitochondria: possible direct function in mitochondrial biogenesis. J Biol Chem 2010; 285: 21590-21599.
9. Badi I, Burba I, Ruggeri C, Zeni F, Bertolotti M, Scopece A, Pompilio G, Raucci A. MicroRNA-34a Induces Vascular Smooth Muscle Cells Senescence by SIRT1 Downregulation and Promotes the Expression of Age-Associated Pro-inflammatory Secretory Factors. J Gerontol A Biol Sci Med Sci 2015; 70: 1304-1311.
10. Bae HJ, Chang YG, Noh JH, Kim JK, Eun JW, Jung KH, Kim MG, Shen Q, Ahn YM, Kwon SH, Park WS, Lee JY, Nam SW. DBC1 does not function as a negative regulator of SIRT1 in liver cancer. Oncol Lett 2012; 4: 873-877.
11. Bai P, Canto C, Brunyánszki A, Huber A, Szántó M, Cen Y, Yamamoto H, Houten SM, Kiss B, Oudart H, Gergely P, Menissier-de Murcia J, Schreiber V, Sauve AA, Auwerx J. PARP-2 regulates SIRT1 expression and whole-body energy expenditure. Cell Metab 2011; 13: 450-460.
12. Bai P, Cantó C, Oudart H, Brunyánszki A, Cen Y, Thomas C, Yamamoto H, Huber A, Kiss B, Houtkooper RH, Schoonjans K, Schreiber V, Sauve AA, Menissier-de Murcia J, Auwerx J. PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. Cell Metab 2011; 13: 461-468.
13. Barber MF, Michishita-Kioi E, Xi Y, Tasselli L, Kioi M, Moqtaderi Z, Tennen RI, Paredes S, Young NL, Chen K, Struhl K, Garcia BA, Gozani O, Li W, Chua KF. SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. Nature 2012; 487: 114-118.
14. Baur JA, Ungvari Z, Minor RK, Couteur DGL, de Cabo R. Are sirtuins viable targets for improving healthspan and lifespan? Nat Rev Drug Discov 2012; 11: 443-461.
15. Bellizzi D, Covello G, Di Cianni F, Tong Q, De Benedictis G. Identification of GATA2 and AP-1 Activator elements within the enhancer VNTR occurring in intron 5 of the human SIRT3 gene. Mol Cells 2009; 28: 87-92.
16. Bellizzi D, Rose G, Cavalcante P, Covello G, Dato S, De Rango F, et al. A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. Genomics 2005; 85: 258-263.
17. Bicker S, Schratt G. Not miR-ly aging: SIRT1 boosts memory via a microRNA-dependent mechanism. Cell Res 2010; 20: 1175-1177.
18. Black JC, Mosley A, Kitada T, Washburn M, Carey M. The SIRT2 deacetylase regulates autoacetylation of p300. Mol Cell 2008; 32: 449-455.
19. Bock FJ, Todorova TT, Chang P. RNA Regulation by Poly(ADP-Ribose) Polymerases. Mol Cell 2015; 58: 959-969.
20. Bonda DJ, Lee H-G, Camins A, Pallàs M, Casadesus G, Smith MA, Zhu X. The sirtuin pathway in ageing and Alzheimer disease: mechanistic and therapeutic considerations. Lancet Neurol 2011; 10: 275-279.
21. Bosch-Presegué L, Vaquero A. Sirtuin-dependent epigenetic regulation in the maintenance of genome integrity. FEBS J 2015; 282: 1745-1767.
22. Bouras T, Fu M, Sauve AA, Wang F, Quong AA, Perkins ND, Hay RT, Gu W, Pestell RG. SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. J Biol Chem 2005; 280: 10264-10276.
23. Braidy N, Poljak A, Grant R, Jayasena T, Mansour H, Chan-Ling T, Smythe G, Sachdev P, Guillemin GJ. Differential expression of sirtuins in the aging rat brain. Front Cell Neurosci 2015; 9: 167.
24. Bräutigam L, Jensen LDE, Poschmann G, Nyström S, Bannenberg S, Dreij K, Lepka K, Prozorovski T, Montano SJ, Aktas O, Uhlén P, Stühler K, Cao Y, Holmgren A, Berndt C. Glutaredoxin regulates vascular development by reversible glutathionylation of sirtuin 1. Proc Natl Acad Sci U S A 2013; 110: 20057-20062.
25. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004; 303: 2011-2015.
26. Buler M, Aatsinki S-M, Izzi V, Uusimaa J, Hakkola J. SIRT5 is under the control of PGC-1α and AMPK and is involved in regulation of mitochondrial energy metabolism. FASEB J 2014; 28: 3225-3237.
27. Caito S, Hwang J-W, Chung S, Yao H, Sundar IK, Rahman I. PARP-1 inhibition does not restore oxidant-mediated reduction in SIRT1 activity. Biochem Biophys Res Commun 2010; 392: 264-270.
28. Cantó C, Sauve AA, Bai P. Crosstalk between poly(ADP-ribose) polymerase and sirtuin enzymes. Mol Aspects Med 2013; 34: 1168-1201.
29. Cardinale A, de Stefano MC, Mollinari C, Racaniello M, Garaci E, Merlo D. Biochemical characterization of sirtuin 6 in the brain and its involvement in oxidative stress response. Neurochem Res 2015; 40: 59-69.
30. Ceolotto G, De Kreutzenberg SV, Cattelan A, Fabricio ASC, Squarcina E, Gion M, Semplicini A, Fadini GP, Avogaro A. Sirtuin 1 stabilization by HuR represses TNF-α- and glucose-induced E-selectin release and endothelial cell adhesiveness in vitro: relevance to human metabolic syndrome. Clin Sci 2014; 127: 449-461.

31. Chen C-J, Fu Y-C, Yu W, Wang W. SIRT3 protects cardiomyocytes from oxidative stress-mediated cell death by activating NF-κB. Biochem Biophys Res Commun 2013; 430: 798-803.
32. Chen J, Zhou Y, Mueller-Steiner S, Chen L-F, Kwon H, Yi S, Mucke L, Gan L. SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. J Biol Chem 2005; 280: 40364-40374.
33. Chen Y, Wang H, Luo G, Dai X. SIRT4 inhibits cigarette smoke extracts-induced mononuclear cell adhesion to human pulmonary microvascular endothelial cells via regulating NF-κB activity. Toxicol Lett 2014; 226: 320-327.
34. Chen Z, Shentu TP, Wen L, Johnson DA, Shyy JY. Regulation of SIRT1 by oxidative stress-responsive miRNAs and a systematic approach to identify its role in the endothelium. Antioxid Redox Signal 2013; 19: 1522-1538.
35. Chiang MC, Cheng YC, Lin KH, Yen CH. PPARγ regulates the mitochondrial dysfunction in human neural stem cells with tumor necrosis factor alpha. Neuroscience 2013; 229: 118-129.
36. Choi SE, Fu T, Seok S, Kim D-H, Yu E, Lee KW, Kang Y, Li X, Kemper B, Kemper JK. Elevated microRNA-34a in obesity reduces NAD+ levels and SIRT1 activity by directly targeting NAMPT. Aging Cell 2013; 12: 1062-1072.
37. Choi SE, Kemper JK. Regulation of SIRT1 by microRNAs. Mol Cells 2013; 36: 385-392.
38. Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT. Multiple roles of HDAC inhibition in neurodegenerative conditions. Trends Neurosci 2009; 32: 591-601.
39. Cieślik M, Czapski GA, Strosznajder JB. The Molecular Mechanism of Amyloid β42 Peptide Toxicity: The Role of Sphingosine Kinase-1 and Mitochondrial Sirtuins. PLoS ONE 2015; 10: e0137193.
40. Ciron C, Zheng L, Bobela W, Knott GW, Leone TC, Kelly DP, Schneider BL. PGC-1α activity in nigral dopamine neurons determines vulnerability to α-synuclein. Acta Neuropathol Commun 2015; 3: 16.
41. Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C, Frye R, Ploegh H, Kessler BM, Sinclair DA. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. Mol Cell 2004; 13: 627-638.
42. Culman J, Zhao Y, Gohlke P, Herdegen T. PPAR-gamma: therapeutic target for ischemic stroke. Trends Pharmacol Sci 2007; 28: 244-249.
43. Czapski GA, Adamczyk A, Strosznajder RP, Strosznajder JB. Expression and activity of PARP family members in the hippocampus during systemic inflammation: their role in the regulation of prooxidative genes. Neurochem Int 2013; 62: 664-673.
44. Ding J, Li M, Wan X, Jin X, Chen S, Yu C, Li Y. Effect of miR-34a in regulating steatosis by targeting PPARα expression in nonalcoholic fatty liver disease. Sci Rep 2015; 5: 13729.
45. Dioum EM, Chen R, Alexander MS, Zhang Q, Hogg RT, Gerard RD, Garcia JA. Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. Science 2009; 324: 1289-1293.
46. Dominy JE, Lee Y, Jedrychowski MP, Chim H, Jurczak MJ, Camporez JP, Ruan HB, Feldman J, Pierce K, Mostoslavsky R, Denu JM, Clish CB, Yang X, Shulman GI, Gygi SP, Puigserver P. The deacetylase Sirt6 activates the acetyltransferase GCN5 and suppresses hepatic gluconeogenesis. Mol Cell 2012; 48: 900-913.
47. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, Kim J, Woo J, Kim JH, Choi BH, He B, Chen W, Zhang S, Cerione RA, Auwerx J, Hao Q, Lin H. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science 2011; 334: 806-809.
48. Duclot F, Meffre J, Jacquet C, Gongora C, Maurice T. Mice knock out for the histone acetyltransferase p300/CREB binding protein-associated factor develop a resistance to amyloid toxicity. Neuroscience 2010; 167: 850-863.
49. Fan W, Fang R, Wu X, Liu J, Feng M, Dai G, Chen G, Wu G. Shear-sensitive microRNA-34a modulates flow-dependent regulation of endothelial inflammation. J Cell Sci 2015; 128: 70-80.
50. Fan W, Luo J. SIRT1 regulates UV-induced DNA repair through deacetylating XPA. Mol Cell 2010; 39: 247-258.
51. Fang EF, Scheibye-Knudsen M, Brace LE, Kassahun H, SenGupta T, Nilsen H, Mitchell JR, Croteau DL, Bohr VA. Defective mitophagy in XPA via PARP-1 hyperactivation and NAD(+)/SIRT1 reduction. Cell 2014; 157: 882-896.
52. Feng Y, Liu T, Dong S-Y, Guo Y-J, Jankovic J, Xu H, Wu YC. Rotenone affects p53 transcriptional activity and apoptosis via targeting SIRT1 and H3K9 acetylation in SH-SY5Y cells. J Neurochem 2015; 134: 668-676.
53. Finley LWS, Haas W, Desquiret-Dumas V, Wallace DC, Procaccio V, Gygi SP, Haigis MC. Succinate dehydrogenase is a direct target of sirtuin 3 deacetylase activity. PLoS ONE 2011; 6: e23295.
54. Franke K, Kalucka J, Mamlouk S, Singh RP, Muschter A, Weidemann A, Iyengar V, Jahn S, Wieczorek K, Geiger K, Muders M, Sykes AM, Poitz DM, Ripich T, Otto T, Bergmann S, Breier G, Baretton G, Fong GH, Greaves DR, Bornstein S, Chavakis T, Fandrey J, Gassmann M, Wielockx B. HIF-1α is a protective factor in conditional PHD2-deficient mice suffering from severe HIF-2α-induced excessive erythropoiesis. Blood 2013; 121: 1436-1445.
55. Frescas D, Valenti L, Accili D. Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J Biol Chem 2005; 280: 20589-20595.
56. Fritz KS, Galligan JJ, Smathers RL, Roede JR, Shearn CT, Reigan P, Petersen DR. 4-Hydroxynonenal inhibits SIRT3 via thiol-specific modification. Chem Res Toxicol 2011; 24: 651-662.
57. Fu T, Seok S, Choi S, Huang Z, Suino-Powell K, Xu HE, Kemper B, Kemper JK. MicroRNA 34a inhibits beige and brown fat formation in obesity in part by suppressing adipocyte fibroblast growth factor 21 signaling and SIRT1 function. Mol Cell Biol 2014; 34: 4130-4142.
58. Fuentes E, Guzmán-Jofre L, Moore-Carrasco R, Palomo I. Role of PPARs in inflammatory processes associated with metabolic syndrome (Review). Mol Med Rep 2013; 8: 1611-1616.
59. Gao J, Wang W-Y, Mao Y-W, Gräff J, Guan J-S, Pan L, Mak G, Kim D, Su SC, Tsai LH. A novel pathway regulates memory and plasticity via SIRT1 and miR-134. Nature 2010; 466: 1105-1109.
60. Gerace E, Scartabelli T, Formentini L, Landucci E, Moroni F, Chiarugi A, Pellegrini-Giampietro DE. Mild activation of poly(ADP-ribose) polymerase (PARP) is neuroprotective in rat hippocampal slice models of ischemic tolerance. Eur J Neurosci 2012; 36: 1993-2005.

61. Ghosh HS, Spencer JV, Ng B, McBurney MW, Robbins PD. Sirt1 interacts with transducin-like enhancer of split-1 to inhibit nuclear factor kappaB-mediated transcription. Biochem J 2007; 408: 105-111.
62. Giannakou ME, Partridge L. The interaction between FOXO and SIRT1: tipping the balance towards survival. Trends Cell Biol 2004; 14: 408-412.
63. Gibson TM, Cotter MA, Cameron NE. Effects of poly(ADP-ribose) polymerase inhibition on dysfunction of non-adrenergic non-cholinergic neurotransmission in gastric fundus in diabetic rats. Nitric Oxide 2006; 15: 344-350.
64. Gonfloni S, Iannizzotto V, Maiani E, Bellusci G, Ciccone S, Diederich M. P53 and Sirt1: routes of metabolism and genome stability. Biochem Pharmacol 2014; 92: 149-156.
65. Grimley R, Polyakova O, Vamathevan J, McKenary J, Hayes B, Patel C, Smith J, Bridges A, Fosberry A, Bhardwaja A, Mouzon B, Chung CW, Barrett N, Richmond N, Modha S, Solari R. Over expression of wild type or a catalytically dead mutant of Sirtuin 6 does not influence NFκB responses. PLoS One 2012; 7: e39847.
66. Guarente L. Sirtuins as potential targets for metabolic syndrome. Nature 2006; 444: 868-874.
67. Günschmann C, Stachelscheid H, Akyüz MD, Schmitz A, Missero C, Brüning JC, Niessen CM. Insulin/IGF-1 controls epidermal morphogenesis via regulation of FoxO-mediated p63 inhibition. Dev Cell 2013; 26: 176-187.
68. Guo X, Williams JG, Schug TT, Li X. DYRK1A and DYRK3 promote cell survival through phosphorylation and activation of SIRT1. J Biol Chem 2010; 285: 13223-13232.
69. Gurha P, Wang T, Larimore AH, Sassi Y, Abreu-Goodger C, Ramirez MO, Reddy AK, Engelhardt S, Taffet GE, Wehrens XH, Entman ML, Rodriguez A. microRNA-22 promotes heart failure through coordinate suppression of PPAR/ERR-nuclear hormone receptor transcription. PLoS One 2013; 8: e75882.
70. Gustin JA, Korgaonkar CK, Pincheira R, Li Q, Donner DB. Akt regulates basal and induced processing of NF-kappaB2 (p100) to p52. J Biol Chem 2006; 281: 16473-16481.
71. Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, Valenzuela DM, Yancopoulos GD, Karow M, Blander G, Wolberger C, Prolla TA, Weindruch R, Alt FW, Guarente L. SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. Cell 2006; 126: 941-954.
72. Han L, Zhou R, Niu J, McNutt MA, Wang P, Tong T. SIRT1 is regulated by a PPAR{γ}-SIRT1 negative feedback loop associated with senescence. Nucleic Acids Res 2010; 38: 7458-7471.
73. Han Y, Jin YH, Kim YJ, Kang BY, Choi HJ, Kim DW, Yeo CY, Lee KY. Acetylation of Sirt2 by p300 attenuates its deacetylase activity. Biochem Biophys Res Commun 2008; 375: 576-580.
74. Hassa PO, Hottiger MO. The functional role of poly(ADP-ribose) polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders. Cell Mol Life Sci 2002; 59: 1534-1553.
75. He J, Hu B, Shi X, Weidert ER, Lu P, Xu M, Huang M, Kelley EE, Xie W. Activation of the aryl hydrocarbon receptor sensitizes mice to nonalcoholic steatohepatitis by deactivating mitochondrial sirtuin deacetylase Sirt3. Mol Cell Biol 2013; 33: 2047-2055.
76. Helenius M, Hänninen M, Lehtinen SK, Salminen A. Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-kappa B. Biochem J 1996; 318 (Pt 2): 603-608.
77. Herbert KJ, Cook AL, Snow ET. SIRT1 modulates miRNA processing defects in p53-mutated human keratinocytes. J Dermatol Sci 2014; 74: 142-149.
78. van der Horst A, Tertoolen LGJ, de Vries-Smits LMM, Frye RA, Medema RH, Burgering BMT. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem 2004; 279: 28873-28879.
79. Hottiger MO, Hassa PO, Lüscher B, Schüler H, Koch-Nolte F. Toward a unified nomenclature for mammalian ADP-ribosyltransferases. Trends Biochem Sci 2010; 35: 208-219.
80. Hu G, Wu Z, Yang F, Zhao H, Liu X, Deng Y, Shi M, Zhao G. Ginsenoside Rd blocks AIF mitochondrio-nuclear translocation and NF-κB nuclear accumulation by inhibiting poly(ADP-ribose) polymerase-1 after focal cerebral ischemia in rats. Neurol Sci 2013; 34: 2101-2106.
81. Huang Z-P, Chen J, Seok HY, Zhang Z, Kataoka M, Hu X, Wang DZ. MicroRNA-22 regulates cardiac hypertrophy and remodeling in response to stress. Circ Res 2013; 112: 1234-1243.
82. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000; 403: 795-800.
83. Ito T, Yagi S, Yamakuchi M. MicroRNA-34a regulation of endothelial senescence. Biochem Biophys Res Commun 2010; 398: 735-740.
84. Izhar L, Adamson B, Ciccia A, Lewis J, Pontano-Vaites L, Leng Y, Liang AC, Westbrook TF, Harper JW, Elledge SJ. A Systematic Analysis of Factors Localized to Damaged Chromatin Reveals PARP-Dependent Recruitment of Transcription Factors. Cell Reports 2015; 11: 1486-1500.
85. Jeong J, Juhn K, Lee H, Kim SH, Min BH, Lee KM, Cho MH, Park GH, Lee KH. SIRT1 promotes DNA repair activity and deacetylation of Ku70. Exp Mol Med 2007; 39: 8-13.
86. Jiang H, Huang Y, Xu H, Sun Y, Han N, Li QF. Hypoxia inducible factor-1α is involved in the neurodegeneration induced by isoflurane in the brain of neonatal rats. J Neurochem 2012; 120: 453-460.
87. Jin YH, Kim YJ, Kim DW, Baek KH, Kang BY, Yeo CY, Lee KY. Sirt2 interacts with 14-3-3 beta/gamma and down-regulates the activity of p53. Biochem Biophys Res Commun 2008; 368: 690-695.
88. Jing E, Gesta S, Kahn CR. SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. Cell Metab 2007; 6: 105-114.
89. Jung HJ, Suh Y. Regulation of IGF-1 signaling by microRNAs. Front Genet 2015; 5: 472.
90. Kaidi A, Weinert BT, Choudhary C, Jackson SP. Human SIRT6 promotes DNA end resection through CtIP deacetylation. Science 2010; 329: 1348-1353.
91. Kalmar-Nagy K, Degrell P, Szabo A, Sumegi K, Wittmann I, Gallyas F, Sumegi B. PARP inhibition attenuates acute kidney allograft rejection by suppressing cell death pathways and activating PI-3K-Akt cascade. PLoS One 2013; 8: e81928.

92. Kanfi Y, Naiman S, Amir G, Peshti V, Zinman G, Nahum L, Bar-Joseph Z, Cohen HY. The sirtuin SIRT6 regulates lifespan in male mice. Nature 2012; 483: 218-221.
93. Kang H, Jung JW, Kim MK, Chung JH. CK2 is the regulator of SIRT1 substrate-binding affinity, deacetylase activity and cellular response to DNA-damage. PLoS One 2009; 4: e6611.
94. Kauppinen A, Suuronen T, Ojala J, Kaarniranta K, Salminen A. Antagonistic crosstalk between NF-κB and SIRT1 in the regulation of inflammation and metabolic disorders. Cell Signal 2013; 25: 1939-1948.
95. Kauppinen TM, Suh SW, Higashi Y, Berman AE, Escartin C, Won SJ, Wang C, Cho SH, Gan L, Swanson RA. Poly(ADP-ribose)polymerase-1 modulates microglial responses to amyloid β. J Neuroinflammation 2011; 8: 152.
96. Kawahara TLA, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KC, Boxer LD, Chang HY, Chua KF. SIRT6 links histone H3 lysine 9 deacetylation to control of NF-κB dependent gene expression and organismal lifespan. Cell 2009; 136: 62-74.
97. Kawahara TLA, Rapicavoli NA, Wu AR, Qu K, Quake SR, Chang HY. Dynamic chromatin localization of Sirt6 shapes stress- and aging-related transcriptional networks. PLoS Genet 2011; 7: e1002153.
98. Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, Delalle I, Baur JA, Sui G, Armour SM, Puigserver P, Sinclair DA, Tsai LH. SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. EMBO J 2007; 26: 3169-3179.
99. Kim EJ, Kho JH, Kang MR, Um SJ. Active regulator of SIRT1 cooperates with SIRT1 and facilitates suppression of p53 activity. Mol Cell 2007; 28: 277-290.
100. Kim MY, Kang ES, Ham SA, Hwang JS, Yoo TS, Lee H, Paek KS, Park C, Lee HT, Kim JH, Han CW, Seo HG. The PPARδ-mediated inhibition of angiotensin II-induced premature senescence in human endothelial cells is SIRT1-dependent. Biochem Pharmacol 2012; 84: 1627-1634.
101. Kobayashi Y, Furukawa-Hibi Y, Chen C, Horio Y, Isobe K, Ikeda K, Motoyama N. SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. Int J Mol Med 2005; 16: 237-243.
102. Kokkola T, Suuronen T, Molnár F, Määttä J, Salminen A, Jarho EM, Lahtela-Kakkonen M. AROS has a context-dependent effect on SIRT1. FEBS Lett 2014; 588: 1523-1528.
103. Kolthur-Seetharam U, Dantzer F, McBurney MW, de Murcia G, Sassone-Corsi P. Control of AIF-mediated cell death by the functional interplay of SIRT1 and PARP-1 in response to DNA damage. Cell Cycle 2006; 5: 873-877.
104. Korhonen P, Kyrylenko S, Suuronen T, Salminen A. Changes in DNA binding pattern of transcription factor YY1 in neuronal degeneration. Neurosci Lett 2005; 377: 121-124.
105. Kovacs K, Toth A, Deres P, Kalai T, Hideg K, Gallyas F, Sumegi B. Critical role of PI3-kinase/Akt activation in the PARP inhibitor induced heart function recovery during ischemia-reperfusion. Biochem Pharmacol 2006; 71: 441-452.
106. Li N, Muthusamy S, Liang R, Sarojini H, Wang E. Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1. Mech Ageing Dev 2011; 132: 75-85.
107. Li P, Zhang L, Zhou C, Lin N, Liu A. Sirt 1 activator inhibits the AGE-induced apoptosis and p53 acetylation in human vascular endothelial cells. J Toxicol Sci 2015; 40: 615-624.
108. Li X, Khanna A, Li N, Wang E. Circulatory miR34a as an RNA-based, noninvasive biomarker for brain aging. Aging (Albany NY) 2011; 3: 985-1002.
109. Li Y, Matsumori H, Nakayama Y, Osaki M, Kojima H, Kurimasa A, Ito H, Mori S, Katoh M, Oshimura M, Inoue T. SIRT2 down-regulation in HeLa can induce p53 accumulation via p38 MAPK activation-dependent p300 decrease, eventually leading to apoptosis. Genes Cells 2011; 16: 34-45.
110. Lim JH, Lee YM, Chun YS, Chen J, Kim JE, Park JW. Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. Mol Cell 2010; 38: 864-878.
111. Lin L, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity 2004; 21: 203-213.
112. Lisachev PD, Pustylnyak VO, Shtark MB. Sirt1 Regulates p53 Stability and Expression of Its Target S100B during Long-Term Potentiation in Rat Hippocampus. Bull Exp Biol Med 2016; 160: 432-434.
113. Liszt G, Ford E, Kurtev M, Guarente L. Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. J Biol Chem 2005; 280: 21313-21320.
114. Liu DJ, Hammer D, Komlos D, Chen KY, Firestein BL, Liu AY-C. SIRT1 knockdown promotes neural differentiation and attenuates the heat shock response. J Cell Physiol 2014; 229: 1224-1235.
115. Liu P, Wilson MJ. miR-520c and miR-373 target mTOR and SIRT1, activate the Ras/Raf/MEK/Erk pathway and NF-κB, with up-regulation of MMP9 in human fibrosarcoma cells. J Cell Physiol 2012; 227: 867-876.
116. Liu R, Fan M, Candas D, Qin L, Zhang X, Eldridge A, Zou JX, Zhang T, Juma S, Jin C, Li RF, Perks J, Sun LQ, Vaughan AT, Hai CX, Gius DR, Li JJ. CDK1-Mediated SIRT3 Activation Enhances Mitochondrial Function and Tumor Radioresistance. Mol Cancer Ther 2015; 14: 2090-2102.
117. Lo Sasso G, Menzies KJ, Mottis A, Piersigilli A, Perino A, Yamamoto H, Schoonjans K, Auwerx J. SIRT2 deficiency modulates macrophage polarization and susceptibility to experimental colitis. PLoS One 2014; 9: e103573.
118. Mahlknecht U, Ho AD, Letzel S, Voelter-Mahlknecht S. Assignment of the NAD-dependent deacetylase sirtuin 5 gene (SIRT5) to human chromosome band 6p23 by in situ hybridization. Cytogenet Genome Res 2006; 112: 208-212.
119. Mahlknecht U, Ho AD, Voelter-Mahlknecht S. Chromosomal organization and fluorescence in situ hybridization of the human Sirtuin 6 gene. Int J Oncol 2006; 28: 447-456.
120. Mahlknecht U, Voelter-Mahlknecht S. Chromosomal characterization and localization of the NAD+-dependent histone deacetylase gene sirtuin 1 in the mouse. Int J Mol Med 2009; 23: 245-252.
121. Mahlknecht U, Voelter-Mahlknecht S. Fluorescence in situ hybridization and chromosomal organization of the sirtuin 4 gene (Sirt4) in the mouse. Biochem Biophys Res Commun 2009; 382: 685-690.

122. Mahlknecht U, Voelter-Mahlknecht S. Genomic organization and localization of the NAD-dependent histone deacetylase gene sirtuin 3 (Sirt3) in the mouse. Int J Oncol 2011; 38: 813-822.
123. Mahlknecht U, Zschoernig B. Involvement of Sirtuins in Life-Span and Aging Related Diseases. In: Sensing in Nature. López-Larrea C (ed.). Springer, New York 2012; pp. 252-261.
124. Malewicz M, Perlmann T. Function of transcription factors at DNA lesions in DNA repair. Exp Cell Res 2014; 329: 94-100.
125. Manolopoulos KN, Klotz L-O, Korsten P, Bornstein SR, Barthel A. Linking Alzheimer's disease to insulin resistance: the FoxO response to oxidative stress. Mol Psychiatry 2010; 15: 1046-1052.
126. Mao Z, Hine C, Tian X, Van Meter M, Au M, Vaidya A, Seluanov A, Gorbunova V. SIRT6 promotes DNA repair under stress by activating PARP1. Science 2011; 332: 1443-1446.
127. McCord RA, Michishita E, Hong T, Berber E, Boxer LD, Kusumoto R, Guan S, Shi X, Gozani O, Burlingame AL, Bohr VA, Chua KF. SIRT6 stabilizes DNA-dependent protein kinase at chromatin for DNA double-strand break repair. Aging (Albany NY) 2009; 1: 109-121.
128. Mehrotra P, Riley JP, Patel R, Li F, Voss L 'erin, Goenka S. PARP-14 Functions as a Transcriptional Switch for Stat6-dependent Gene Activation. J Biol Chem 2011; 286: 1767-1776.
129. Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, Vasa-Nicotera M, Ippoliti A, Novelli G, Melino G, Lauro R, Federici M. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. Circulation 2009; 120: 1524-1532.
130. Mester L, Szabo A, Atlasz T, Szabadfi K, Reglodi D, Kiss P, Racz B, Tamas A, Gallyas F Jr, Sumegi B, Hocsak E, Gabriel R, Kovacs K. Protection against chronic hypoperfusion-induced retinal neurodegeneration by PARP inhibition via activation of PI-3-kinase Akt pathway and suppression of JNK and p38 MAP kinases. Neurotox Res 2009; 16: 68-76.
131. Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TL, Barrett JC, Chang HY, Bohr VA, Ried T, Gozani O, Chua KF. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature 2008; 452: 492-496.
132. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell 2005; 16: 4623-4635.
133. Milton VJ, Sweeney ST. Oxidative stress in synapse development and function. Dev Neurobiol 2012; 72: 100-110.
134. Min L, Ji Y, Bakiri L, Qiu Z, Cen J, Chen X, Chen L, Scheuch H, Zheng H, Qin L, Zatloukal K, Hui L, Wagner EF. Liver cancer initiation is controlled by AP-1 through SIRT6-dependent inhibition of survivin. Nat Cell Biol 2012; 14: 1203-1211.
135. Ming M, Shea CR, Guo X, Li X, Soltani K, Han W, He YY. Regulation of global genome nucleotide excision repair by SIRT1 through xeroderma pigmentosum C. Proc Natl Acad Sci U S A 2010; 107: 22623-22628.
136. Mitchell SJ, Martin-Montalvo A, Mercken EM, Palacios HH, Ward TM, Abulwerdi G, Minor RK, Vlasuk GP, Ellis JL, Sinclair DA, Dawson J, Allison DB, Zhang Y, Becker KG, Bernier M, de Cabo R. The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet. Cell Rep 2014; 6: 836-843.
137. Mohamed JS, Hajira A, Pardo PS, Boriek AM. MicroRNA-149 inhibits PARP-2 and promotes mitochondrial biogenesis via SIRT-1/PGC-1α network in skeletal muscle. Diabetes 2014; 63: 1546-1559.
138. Montero J, Dutta C, van Bodegom D, Weinstock D, Letai A. p53 regulates a non-apoptotic death induced by ROS. Cell Death Differ 2013; 20: 1465-1474.
139. Moran EP, Ma J-X. Therapeutic Effects of PPAR α on Neuronal Death and Microvascular Impairment. PPAR Res 2015; 2015: 595426.
140. Nagara Y, Tateishi T, Yamasaki R, Hayashi S, Kawamura M, Kikuchi H, Iinuma KM, Tanaka M, Iwaki T, Matsushita T, Ohyagi Y, Kira J. Impaired cytoplasmic-nuclear transport of hypoxia-inducible factor-1α in amyotrophic lateral sclerosis. Brain Pathol 2013; 23: 534-546.
141. Nahálková J. Novel protein-protein interactions of TPPII, p53, and SIRT7. Mol Cell Biochem 2015; 409: 13-22.
142. Nasrabady SE, Kuzhandaivel A, Akrami A, Bianchetti E, Milanese M, Bonanno G, Nistri A. Unusual increase in lumbar network excitability of the rat spinal cord evoked by the PARP-1 inhibitor PJ-34 through inhibition of glutamate uptake. Neuropharmacology 2012; 63: 415-426.
143. Nasrin N, Kaushik VK, Fortier E, Wall D, Pearson KJ, de Cabo R, Bordone L. JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. PLoS One 2009; 4: e8414.
144. Nemoto S, Fergusson MM, Finkel T. Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. Science 2004; 306: 2105-2108.
145. Nemoto S, Fergusson MM, Finkel T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. J Biol Chem 2005; 280: 16456-16460.
146. Nicolai S, Rossi A, Di Daniele N, Melino G, Annicchiarico-Petruzzelli M, Raschellà G. DNA repair and aging: the impact of the p53 family. Aging (Albany NY) 2015; 7: 1050-1065.
147. Oei SL, Shi Y. Transcription factor Yin Yang 1 stimulates poly(ADP-ribosyl)ation and DNA repair. Biochem Biophys Res Commun 2001; 284: 450-454.
148. Oei SL, Shi Y. Poly(ADP-ribosyl)ation of transcription factor Yin Yang 1 under conditions of DNA damage. Biochem Biophys Res Commun 2001; 285: 27-31.
149. Oka S, Alcendor R, Zhai P, Park JY, Shao D, Cho J, Yamamoto T, Tian B, Sadoshima J. PPARα-Sirt1 complex mediates cardiac hypertrophy and failure through suppression of the ERR transcriptional pathway. Cell Metab 2011; 14: 598-611.
150. Oka S, Zhai P, Yamamoto T, Ikeda Y, Byun J, Hsu CP, Sadoshima J. Peroxisome Proliferator Activated Receptor-α Association With Silent Information Regulator 1 Suppresses Cardiac Fatty Acid Metabolism in the Failing Heart. Circ Heart Fail 2015; 8: 1123-1132.
151. Okawara M, Katsuki H, Kurimoto E, Shibata H, Kume T, Akaike A. Resveratrol protects dopaminergic neurons in midbrain slice culture from multiple insults. Biochem Pharmacol 2007; 73: 550-560.
152. Park J, Chen Y, Tishkoff DX, Peng C, Tan M, Dai L, Xie Z, Zhang Y, Zwaans BM, Skinner ME, Lombard DB, Zhao Y. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. Mol Cell 2013; 50: 919-930.

153. Peck B, Chen CY, Ho KK, Fruscia PD, Myatt SS, Coombes RC, Fuchter MJ, Hsiao CD, Lam EW. SIRT Inhibitors Induce Cell Death and p53 Acetylation through Targeting Both SIRT1 and SIRT2. Mol Cancer Ther 2010; 9: 844-855.
154. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 2007; 8: 49-62.
155. Peserico A, Chiacchiera F, Grossi V, Matrone A, Latorre D, Simonatto M, Fusella A, Ryall JG, Finley LW, Haigis MC, Villani G, Puri PL, Sartorelli V, Simone C. A novel AMPK-dependent FoxO3A-SIRT3 intramitochondrial complex sensing glucose levels. Cell Mol Life Sci 2013; 70: 2015-2029.
156. Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 2004; 429: 771-776.
157. Pillai JB, Isbatan A, Imai S, Gupta MP. Poly(ADP-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD+ depletion and reduced Sir2alpha deacetylase activity. J Biol Chem 2005; 280: 43121-43130.
158. Pillai VB, Sundaresan NR, Gupta MP. Regulation of Akt signaling by Sirtuins: Its implication in cardiac hypertrophy and aging. Circ Res 2014; 114: 368-378.
159. Planavila A, Iglesias R, Giralt M, Villarroya F. Sirt1 acts in association with PPARα to protect the heart from hypertrophy, metabolic dysregulation, and inflammation. Cardiovasc Res 2011; 90: 276-284.
160. Priami C, De Michele G, Cotelli F, Cellerino A, Giorgio M, Pelicci PG, Migliaccio E. Modelling the p53/p66Shc Aging Pathway in the Shortest Living Vertebrate Nothobranchius Furzeri. Aging Dis 2015; 6: 95-108.
161. Quintanilla RA, Utreras E, Cabezas-Opazo FA. Role of PPARγ in the Differentiation and Function of Neurons. PPAR Res 2014; 2014: 768594.
162. Raivich G, Behrens A. Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain. Prog Neurobiol 2006; 78: 347-363.
163. Rajamohan SB, Pillai VB, Gupta M, Sundaresan NR, Birukov KG, Samant S, Hottiger MO, Gupta MP. SIRT1 promotes cell survival under stress by deacetylation-dependent deactivation of poly(ADP-ribose) polymerase 1. Mol Cell Biol 2009; 29: 4116-4129.
164. Ramakrishnan G, Davaakhuu G, Kaplun L, Chung WC, Rana A, Atfi A, Miele L, Tzivion G. Sirt2 deacetylase is a novel AKT binding partner critical for AKT activation by insulin. J Biol Chem 2014; 289: 6054-6066.
165. Ramanan S, Kooshki M, Zhao W, Hsu F-C, Riddle DR, Robbins ME. The PPARalpha agonist fenofibrate preserves hippocampal neurogenesis and inhibits microglial activation after whole-brain irradiation. Int J Radiat Oncol Biol Phys 2009; 75: 870-877.
166. Ramanan VK, Saykin AJ. Pathways to neurodegeneration: mechanistic insights from GWAS in Alzheimer's disease, Parkinson's disease, and related disorders. Am J Neurodegener Dis 2013; 2: 145-175.
167. Rebollo A, Roglans N, Baena M, Sánchez RM, Merlos M, Alegret M, Laguna JC. Liquid fructose downregulates Sirt1 expression and activity and impairs the oxidation of fatty acids in rat and human liver cells. Biochim Biophys Acta 2014; 1841: 514-524.
168. Ren JH, Tao Y, Zhang ZZ, Chen WX, Cai XF, Chen K, Ko BC, Song CL, Ran LK, Li WY, Huang AL, Chen J. Sirtuin 1 regulates hepatitis B virus transcription and replication by targeting transcription factor AP-1. J Virol 2014; 88: 2442-2451.
169. Ren Y, Shan TZ, Zhu LN, Wu T, Guo J, Wang YZ. Effect of breed on the expression of Sirtuins (Sirt1-7) and antioxidant capacity in porcine brain. Animal 2013; 7: 1994-1998.
170. Rodriguez-Ortiz CJ, Baglietto-Vargas D, Martinez-Coria H, LaFerla FM, Kitazawa M. Upregulation of miR-181 decreases c-Fos and SIRT-1 in the hippocampus of 3xTg-AD mice. J Alzheimers Dis 2014; 42: 1229-1238.
171. Rokavec M, Li H, Jiang L, Hermeking H. The p53/miR-34 axis in development and disease. J Mol Cell Biol 2014; 6: 214-230.
172. Sakamaki J, Daitoku H, Yoshimochi K, Miwa M, Fukamizu A. Regulation of FOXO1-mediated transcription and cell proliferation by PARP-1. Biochem Biophys Res Commun 2009; 382: 497-502.
173. Salminen A, Ojala J, Huuskonen J, Kauppinen A, Suuronen T, Kaarniranta K. Interaction of aging-associated signaling cascades: inhibition of NF-kappaB signaling by longevity factors FoxOs and SIRT1. Cell Mol Life Sci 2008; 65: 1049-1058.
174. Salvati E, Scarsella M, Porru M, Rizzo A, Iachettini S, Tentori L, Graziani G, D'Incalci M, Stevens MF, Orlandi A, Passeri D, Gilson E, Zupi G, Leonetti C, Biroccio A. PARP1 is activated at telomeres upon G4 stabilization: possible target for telomere-based therapy. Oncogene 2010; 29: 6280-6293.
175. Saunders LR, Sharma AD, Tawney J, Nakagawa M, Okita K, Yamanaka S, Willenbring H, Verdin E. miRNAs regulate SIRT1 expression during mouse embryonic stem cell differentiation and in adult mouse tissues. Aging (Albany NY) 2010; 2: 415-431.
176. Sayed D, Abdellatif M. AKT-ing via microRNA. Cell Cycle 2010; 9: 3213-3217.
177. Sbodio JI, Lodish HF, Chi NW. Tankyrase-2 oligomerizes with tankyrase-1 and binds to both TRF1 (telomere-repeat-binding factor 1) and IRAP (insulin-responsive aminopeptidase). Biochem J 2002; 361: 451-459.
178. Scheibye-Knudsen M, Croteau DL, Bohr VA. Mitochondrial deficiency in Cockayne syndrome. Mech Ageing Dev 2013; 134: 275-283.
179. Schiltz RL, Nakatani Y. The PCAF acetylase complex as a potential tumor suppressor. Biochim Biophys Acta 2000; 1470: M37-M53.
180. Sharma A, Diecke S, Zhang WY, Lan F, He C, Mordwinkin NM, Chua KF, Wu JC. The role of SIRT6 protein in aging and reprogramming of human induced pluripotent stem cells. J Biol Chem 2013; 288: 18439-18447.
181. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002; 4: E131-136.
182. Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem 2005; 280: 13560-13567.
183. Siddiqui A, Chinta SJ, Mallajosyula JK, Rajagopolan S, Hanson I, Rane A, Melov S, Andersen JK. Selective binding of nuclear alpha-synuclein to the PGC1alpha promoter under conditions of oxidative stress may contribute to losses in mitochondrial function: implications for Parkinson's disease. Free Radic Biol Med 2012; 53: 993-1003.
184. Smit-McBride Z, Forward KI, Nguyen AT, Bordbari MH, Oltjen SL, Hjelmeland LM. Age-dependent increase in miRNA-34a

expression in the posterior pole of the mouse eye. Mol Vis 2014; 20: 1569-1578.
185. Song Y, Shi J, Wu Y, Han C, Zou J, Shi Y, Liu Z. Metformin ameliorates insulin resistance in L6 rat skeletal muscle cells through upregulation of SIRT3. Chin Med J 2014; 127: 1523-1529.
186. Song ZF, Chen DY, Du B, Ji XP. Poly (ADP-ribose) polymerase inhibitor reduces heart ischaemia/reperfusion injury via inflammation and Akt signalling in rats. Chin Med J 2013; 126: 1913-1917.
187. Strosznajder JB, Jeśko H, Strosznajder RP. Effect of amyloid beta peptide on poly(ADP-ribose) polymerase activity in adult and aged rat hippocampus. Acta Biochim Pol 2000; 47: 847-854.
188. Strosznajder RP, Czubowicz K, Jesko H, Strosznajder JB. Poly(ADP-ribose) metabolism in brain and its role in ischemia pathology. Mol Neurobiol 2010; 41: 187-196.
189. Strosznajder RP, Jesko H, Adamczyk A. Effect of aging and oxidative/genotoxic stress on poly(ADP-ribose) polymerase-1 activity in rat brain. Acta Biochim Pol 2005; 52: 909-914.
190. Strosznajder RP, Jesko H, Zambrzycka A. Poly(ADP-ribose) polymerase: the nuclear target in signal transduction and its role in brain ischemia-reperfusion injury. Mol Neurobiol 2005; 31: 149-167.
191. Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, Alford A, Waters KM. MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. Mol Endocrinol 2009; 23: 1876-1884.
192. Sundaresan NR, Pillai VB, Wolfgeher D, Samant S, Vasudevan P, Parekh V, Raghuraman H, Cunningham JM, Gupta M, Gupta MP. The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. Sci Signal 2011; 4: ra46.
193. Sundaresan NR, Vasudevan P, Zhong L, Kim G, Samant S, Parekh V, Pillai VB, Ravindra PV, Gupta M, Jeevanandam V, Cunningham JM, Deng CX, Lombard DB, Mostoslavsky R, Gupta MP. The sirtuin SIRT6 blocks IGF-Akt signaling and development of cardiac hypertrophy by targeting c-Jun. Nat Med 2012; 18: 1643-1650.
194. Takasaka N, Araya J, Hara H, Ito S, Kobayashi K, Kurita Y, Wakui H, Yoshii Y, Yumino Y, Fujii S, Minagawa S, Tsurushige C, Kojima J, Numata T, Shimizu K, Kawaishi M, Kaneko Y, Kamiya N, Hirano J, Odaka M, Morikawa T, Nishimura SL, Nakayama K, Kuwano K. Autophagy induction by SIRT6 through attenuation of insulin-like growth factor signaling is involved in the regulation of human bronchial epithelial cell senescence. J Immunol 2014; 192: 958-968.
195. Takizawa Y, Nakata R, Fukuhara K, Yamashita H, Kubodera H, Inoue H. The 4'-hydroxyl group of resveratrol is functionally important for direct activation of PPARα. PLoS One 2015; 10: e0120865.
196. Tao Y, Huang C, Huang Y, Hong L, Wang H, Zhou Z, Qiu Y. SIRT4 Suppresses Inflammatory Responses in Human Umbilical Vein Endothelial Cells. Cardiovasc Toxicol 2015; 15: 217-223.
197. Tong L, Denu JM. Function and metabolism of sirtuin metabolite O-acetyl-ADP-ribose. Biochim Biophys Acta 2010; 1804: 1617-1625.
198. Tsai Y-C, Greco TM, Boonmee A, Miteva Y, Cristea IM. Functional proteomics establishes the interaction of SIRT7 with chromatin remodeling complexes and expands its role in regulation of RNA polymerase I transcription. Mol Cell Proteomics 2012; 11: 60-76.
199. Tulin A, Stewart D, Spradling AC. The Drosophila heterochromatic gene encoding poly(ADP-ribose) polymerase (PARP) is required to modulate chromatin structure during development. Genes Dev 2002; 16: 2108-2119.
200. Valentin-Vega YA, Maclean KH, Tait-Mulder J, Milasta S, Steeves M, Dorsey FC, Cleveland JL, Green DR, Kastan MB. Mitochondrial dysfunction in ataxia-telangiectasia. Blood 2012; 119: 1490-1500.
201. Vélez-Cruz R, Johnson DG. E2F1 and p53 Transcription Factors as Accessory Factors for Nucleotide Excision Repair. Int J Mol Sci 2012; 13: 13554-13568.
202. Voelter-Mahlknecht S, Ho AD, Mahlknecht U. FISH-mapping and genomic organization of the NAD-dependent histone deacetylase gene, Sirtuin 2 (Sirt2). Int J Oncol 2005; 27: 1187-1196.
203. Voelter-Mahlknecht S, Letzel S, Mahlknecht U. Fluorescence in situ hybridization and chromosomal organization of the human Sirtuin 7 gene. Int J Oncol 2006; 28: 899-908.
204. Volonte D, Zou H, Bartholomew JN, Liu Z, Morel PA, Galbiati F. Oxidative Stress-induced Inhibition of Sirt1 by Caveolin-1 Promotes p53-dependent Premature Senescence and Stimulates the Secretion of Interleukin 6 (IL-6). J Biol Chem 2015; 290: 4202-4214.
205. Vyas S, Matic I, Uchima L, Rood J, Zaja R, Hay RT, Ahel I, Chang P. Family-wide analysis of poly(ADP-ribose) polymerase activity. Nat Commun 2014; 5: 4426.
206. Wang C, Chen L, Hou X, Li Z, Kabra N, Ma Y, Nemoto S, Finkel T, Gu W, Cress WD, Chen J. Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. Nat Cell Biol 2006; 8: 1025-1031.
207. Wang F, Nguyen M, Qin FX-F, Tong Q. SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. Aging Cell 2007; 6: 505-514.
208. Wang F, Tong Q. SIRT2 Suppresses Adipocyte Differentiation by Deacetylating FOXO1 and Enhancing FOXO1's Repressive Interaction with PPARγ. Mol Biol Cell 2009; 20: 801-808.
209. Wang HF, Li Q, Feng RL, Wen TQ. Transcription levels of sirtuin family in neural stem cells and brain tissues of adult mice. Cell Mol Biol (Noisy-le-grand) 2012; Suppl 58: OL1737-1743.
210. Wang J, Fivecoat H, Ho L, Pan Y, Ling E, Pasinetti GM. The role of Sirt1: at the crossroad between promotion of longevity and protection against Alzheimer's disease neuropathology. Biochim Biophys Acta 2010; 1804: 1690-1694.
211. Wang R, Li JJ, Diao S, Kwak YD, Liu L, Zhi L, Büeler H, Bhat NR, Williams RW, Park EA, Liao FF. Metabolic stress modulates Alzheimer's β-secretase gene transcription via SIRT1-PPARγ-PGC-1 in neurons. Cell Metab 2013; 17: 685-694.
212. Wang S, Yang X, Lin Y, Qiu X, Li H, Zhao X, Cao L, Liu X, Pang Y, Wang X, Chi Z. Cellular NAD depletion and decline of SIRT1 activity play critical roles in PARP-1-mediated acute epileptic neuronal death in vitro. Brain Res 2013; 1535: 14-23.
213. Wang WR, Liu EQ, Zhang JY, Li YX, Yang XF, He YH, Zhang W, Jing T, Lin R. Activation of PPAR alpha by fenofibrate inhibits apoptosis in vascular adventitial fibroblasts partly through SIRT1-mediated deacetylation of FoxO1. Exp Cell Res 2015; 338: 54-63.

214. Wang Y, Pang WJ, Wei N, Xiong Y, Wu WJ, Zhao CZ, Shen QW, Yang GS. Identification, stability and expression of Sirt1 antisense long non-coding RNA. Gene 2014; 539: 117-124.
215. Wang Y, Zhao X, Shi D, Chen P, Yu Y, Yang L, Xie L. Overexpression of SIRT1 promotes high glucose-attenuated corneal epithelial wound healing via p53 regulation of the IGFBP3/IGF-1R/AKT pathway. Invest Ophthalmol Vis Sci 2013; 54: 3806-3814.
216. Wesierska-Gadek J, Wojciechowski J, Schmid G. Phosphorylation regulates the interaction and complex formation between wt p53 protein and PARP-1. J Cell Biochem 2003; 89: 1260-1284.
217. Wu M, Seto E, Zhang J. E2F1 enhances glycolysis through suppressing Sirt6 transcription in cancer cells. Oncotarget 2015; 6: 11252-11263.
218. Wu Y, Chen L, Wang Y, Li W, Lin Y, Yu D, et al. Overexpression of Sirtuin 6 suppresses cellular senescence and NF-κB mediated inflammatory responses in osteoarthritis development. Sci Rep 2015; 5: 17602.
219. Xiong S, Salazar G, Patrushev N, Alexander RW. FoxO1 mediates an autofeedback loop regulating SIRT1 expression. J Biol Chem 2011; 286: 5289-5299.
220. Xu J, Li L, Yun H, Han Y. MiR-138 promotes smooth muscle cells proliferation and migration in db/db mice through down-regulation of SIRT1. Biochem Biophys Res Commun 2015; 463: 1159-1164.
221. Yakunin E, Kisos H, Kulik W, Grigoletto J, Wanders RJA, Sharon R. The regulation of catalase activity by PPAR γ is affected by α-synuclein. Ann Clin Transl Neurol 2014; 1: 145-159.
222. Yamamori T, DeRicco J, Naqvi A, Hoffman TA, Mattagajasingh I, Kasuno K, Jung SB, Kim CS, Irani K. SIRT1 deacetylates APE1 and regulates cellular base excision repair. Nucleic Acids Res 2010; 38: 832-845.
223. Yan J, Huang Y, Lu Y, Chen J, Jiang H. Repeated administration of ketamine can induce hippocampal neurodegeneration and long-term cognitive impairment via the ROS/HIF-1α pathway in developing rats. Cell Physiol Biochem 2014; 33: 1715-1732.
224. Yang H, Yan B, Liao D, Huang S, Qiu Y. Acetylation of HDAC1 and degradation of SIRT1 form a positive feedback loop to regulate p53 acetylation during heat-shock stress. Cell Death Dis 2015; 6: e1747.
225. Yang SJ, Choi JM, Chae SW, Kim WJ, Park SE, Rhee EJ, Lee WY, Oh KW, Park SW, Kim SW, Park CY. Activation of peroxisome proliferator-activated receptor gamma by rosiglitazone increases sirt6 expression and ameliorates hepatic steatosis in rats. PLoS One 2011; 6: e17057.
226. Yang T, Chen X, Jin H, Sethi G, Go ML. Functionalized tetrahydro-1H-pyrido[4,3-b]indoles: A novel chemotype with Sirtuin 2 inhibitory activity. Eur J Med Chem 2015; 92: 145-155.
227. Yang Y, Fu W, Chen J, Olashaw N, Zhang X, Nicosia SV, Bhalla K, Bai W. SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. Nat Cell Biol 2007; 9: 1253-1262.
228. Yang Y, Hou H, Haller EM, Nicosia SV, Bai W. Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. EMBO J 2005; 24: 1021-1032.
229. Ye Z, Fang J, Dai S, Wang Y, Fu Z, Feng W, Wei Q, Huang P. MicroRNA-34a induces a senescence-like change via the down-regulation of SIRT1 and up-regulation of p53 protein in human esophageal squamous cancer cells with a wild-type p53 gene background. Cancer Lett 2016; 370: 216-221.
230. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J 2004; 23: 2369-2380.
231. Yin H, Liang X, Jogasuria A, Davidson NO, You M. miR-217 regulates ethanol-induced hepatic inflammation by disrupting sirtuin 1-lipin-1 signaling. Am J Pathol 2015; 185: 1286-1296.
232. Yuan F, Xie Q, Wu J, Bai Y, Mao B, Dong Y, Bi W, Ji G, Tao W, Wang Y, Yuan Z. MST1 promotes apoptosis through regulating Sirt1-dependent p53 deacetylation. J Biol Chem 2011; 286: 6940-6945.
233. Zhang E, Guo Q, Gao H, Xu R, Teng S, Wu Y. Metformin and Resveratrol Inhibited High Glucose-Induced Metabolic Memory of Endothelial Senescence through SIRT1/p300/p53/p21 Pathway. PLoS ONE 2015; 10: e0143814.
234. Zhang F, Wang S, Gan L, Vosler PS, Gao Y, Zigmond MJ, et al. Protective effects and mechanisms of sirtuins in the nervous system. Prog Neurobiol 2011; 95: 373-395.
235. Zhang P, Tu B, Wang H, Cao Z, Tang M, Zhang C, et al. Tumor suppressor p53 cooperates with SIRT6 to regulate gluconeogenesis by promoting FoxO1 nuclear exclusion. Proc Natl Acad Sci USA 2014; 111: 10684-10689.
236. Zhang R, Chen H-Z, Liu J-J, Jia Y-Y, Zhang Z-Q, Yang R-F, et al. SIRT1 suppresses activator protein-1 transcriptional activity and cyclooxygenase-2 expression in macrophages. J Biol Chem 2010; 285: 7097-7110.
237. Zhao K, Zhou Y, Qiao C, Ni T, Li Z, Wang X, et al. Oroxylin A promotes PTEN-mediated negative regulation of MDM2 transcription via SIRT3-mediated deacetylation to stabilize p53 and inhibit glycolysis in wt-p53 cancer cells. J Hematol Oncol 2015; 8: 41.
238. Zhao W, Zhao J, Hou M, Wang Y, Zhang Y, Zhao X, et al. HuR and TIA1/TIAL1 are involved in regulation of alternative splicing of SIRT1 pre-mRNA. Int J Mol Sci 2014; 15: 2946-2958.
239. Zheng B, Liao Z, Locascio JJ, Lesniak KA, Roderick SS, Watt ML, et al. PGC-1α, A Potential Therapeutic Target for Early Intervention in Parkinson's Disease. Sci Transl Med 2010; 2: 52ra73.
240. Zheng T, Lu Y. SIRT1 Protects Human Lens Epithelial Cells Against Oxidative Stress by Inhibiting p53-Dependent Apoptosis. Curr Eye Res 2015; : 1-8.
241. Zheng Y, Xu Z. MicroRNA-22 induces endothelial progenitor cell senescence by targeting AKT3. Cell Physiol Biochem 2014; 34: 1547-1555.
242. Zhong L, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, et al. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. Cell 2010; 140: 280-293.

Original paper

Folia Neuropathologica

5-Aminolevulinic acid-mediated sonosensitization of rat RG2 glioma cells *in vitro*

Krzysztof Bilmin[1], Tamara Kujawska[2], Wojciech Secomski[2], Andrzej Nowicki[2], Paweł Grieb[1]

[1]Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, [2]Institute of Fundamental Technological Research, Polish Academy of Sciences, Warsaw, Poland

Folia Neuropathol 2016; 54 (3): 234-240 DOI: 10.5114/fn.2016.62233

Abstract

Sonodynamic therapy (SDT) is a promising technique based on the ability of certain substances, called sonosensitizers, to sensitize cancer cells to non-thermal effects of low-energy ultrasound waves, allowing their destruction. Sonosensitization is thought to induce cell death by direct physical effects such as cavitation and acoustical streaming as well as by complementary chemical reactions generating oxygen free radicals. One of the promising sonosensitizers is 5-aminolevulinic acid (ALA) which upon selective uptake by cancer cells is metabolized and accumulated as protoporphyrin IX. The objective of the study was to describe ALA-mediated sonodynamic effects in vitro on a rat RG2 glioma cell line. Glioma cells, seeded at the bottom of 96-well plates and incubated with ALA (10 µg/ml) for 6 h, were exposed to the sinusoidal US pulses with a resonance frequency of 1 MHz, 1000 µs duration, 0.4 duty-cycle, and average acoustic power varying from 2 W to 6 W. Ultrasound waves were generated by a flat circular piezoelectric transducer with a diameter of 25 mm. Cell viability was determined by MTT assay. Structural cellular changes were visualized with a fluorescence microscope. Signs of cytotoxicity such as a decrease in cell viability, chromatin condensation and apoptosis were found. ALA-mediated SDT evokes cytotoxic effects of low intensity US on rat RG2 glioma cells in vitro. This cell line is indicated for further preclinical assessment of SDT in in vivo conditions.

Key words: *5-aminolevulinic acid, sonodynamic therapy, rat RG2 glioma cells, cell viability.*

Introduction

Sonodynamic therapy (SDT) is a new treatment modality of solid cancers in the early preclinical development phase. The idea of SDT stems from photodynamic therapy (PDT) in which photo-sensitizer substances excited by light produce the avalanche of cytotoxic reactive oxygen species that kill cancer cells. Unlike PDT, SDT uses low-intensity ultrasound (US) waves to kill cells. A low-intensity ultrasound may be defined as US which does not produce hyperthermia which would be directly cytotoxic (> 43°C). To obtain cell killing by low-intensity US it is necessary to expose cells to a sonosensitizer, i.e. a substance that displays a property of sensitizing cells to US. Many photosensitizers, for example hematoporphyrin and its derivatives, act also as sonosensitizers. Low-intensity sonication of cells causes cavitation and other direct acoustic effects that, in the presence of a sonosensitizer, evoke cytotoxic oxygen free radicals. An ideal sonosensitizer should be preferentially taken up and retained

Communicating author

Paweł Grieb, Mossakowski Medical Research Centre, Polish Academy of Sciences, Pawinskiego 5, 02-106 Warsaw, Poland, phone: +48 22 608 65 27, e-mail: pgrieb@imdik.pan.pl

by cancer cells, and display no significant toxicity toward normal tissue. Unlike PDT, which due to limited penetration of light through tissues is applicable only to superficially located tumors, SDT might be used to treat deeply seated cancers [12,16].

One of the photosensitizers used in PDT and potentially useful also for SDT is 5-aminolevulinic acid (ALA), a natural precursor of protoporphyrin IX (PpIX). Due to peculiar metabolic abnormality usually associated with cancer, PpIX is preferentially accumulated in cancer cells, in particular in cells of malignant gliomas, therefore ALA is capable of sensitizing them selectively [4,13]. Development of SDT is particularly awaited for the most malignant glioma, glioblastoma multiforme, which infiltrate brain, cannot be totally removed by surgery and escape radio- and chemotherapy, therefore it recurs and in the majority of cases is lethal within less than 2 years [3].

In the previous study [8] we developed an experimental arrangement for investigating effects of sonication on glioma cells *in vitro* and used this system to determine a relationship between US energy delivered to the rat C6 glioma cells *in vitro* and their vitality; we also established the threshold exposure time that does not induce thermal effects which would be directly cytotoxic. The next step in the translational development of SDT for gliomas should be experiments with glioma cells implanted orthotopically into brains of experimental animals. C6 rat glioma cells implanted to the rat brain have been extensively used as a rat model of human malignant gliomas, but these cells evoke immune response which restricts their infiltrative growth [1]. Therefore for further studies we chose the RG2 rat glioma cell line which is not immunogenic when implanted to rats of Fisher or Wistar strain and displays a highly infiltrative pattern of growth, reminiscent of human glioblastoma multiforme [15].

Material and methods

Cell culture and reagents

The rat RG2 glioma cells were obtained from the cell bank of the American Type Culture Collection (Manassas, VA). The cells were cultured in Petri's dishes in a Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies Inc., USA) supplemented with a 10% fetal bovine serum (FBS, Hyclone, USA) and 1% antibiotics (penicillin and streptomycin). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2/95% air in incubator (Esco). In each experiment cells were used after 24 h of growth.

5-Aminolevulinic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and other chemicals used were purchased from Sigma-Aldrich (Germany), unless specified otherwise.

Experimental set-up for sonodynamic therapy

To examine the impact of ALA-mediated SDT therapy on the tested cells *in vitro* the experimental setup shown in Figure 1 was used.

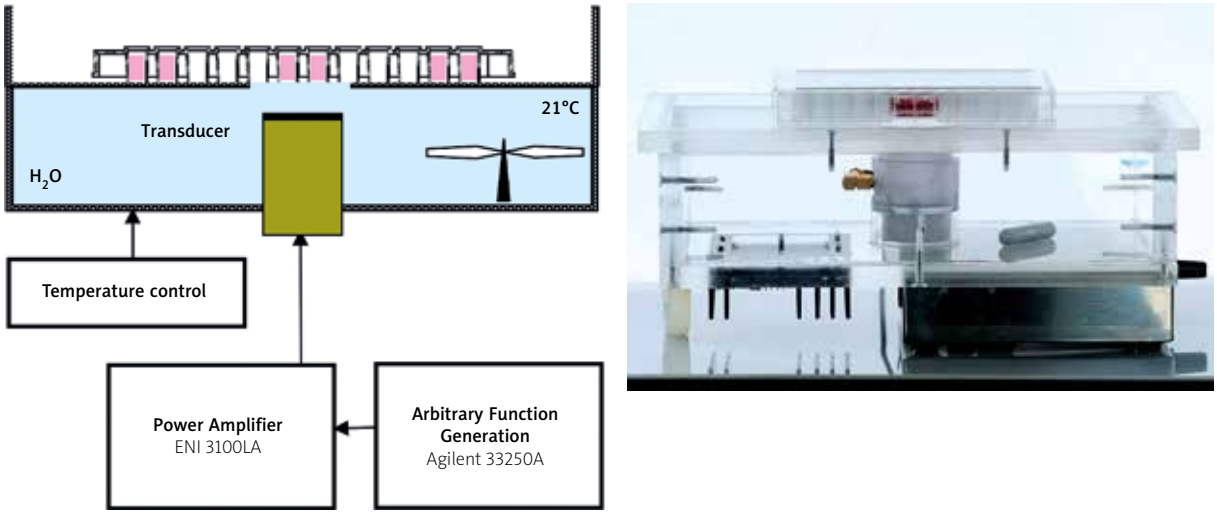


Fig. 1. The experimental arrangement (left) for sonification of rat RG2 glioma cells and its photo (right).

To eliminate the temperature rise in the region of cells caused by the overheating of the US source, the transducer was mounted in the water bath coaxially with wells. The cells cultured on the bottom of wells were sonicated from the bottom of 96-well plates submerged in the water bath, with temperature set to 21°C.

Ultrasonic waves were generated with a planar circular transducer made of a power piezoceramics Pz28 (Meggitt, Kvistgaard, Denmark) with the resonance frequency of 1 MHz and diameter of 25 mm. The transducer had neither a back load nor a quarterwavelength matching layer and was excited by 1000-cycle sinusoidal pulses with a resonance frequency, 0.4 duty-cycle and varied voltage. The electrical pulses were generated by an arbitrary function generator Agilent 33250 (Colorado Springs, USA) and amplified with a power amplifier ENI 3100L (ENI, Rochester, NY, USA). The average acoustic power of the generated beam was varied from 2 W to 6 W at 2 W (initial intensity I_{SATA} was varied between 0.5 and 1.5 W/cm^2) and measured using Ultrasound Power Meter UPM-DT-10AV (Ohmic Instruments Co., Easton, USA). These specific ultrasound field parameters were selected to induce no thermal lethality of the cells tested.

Determination of acoustic parameters of pulsed ultrasonic beams

The acoustic properties of the generated pulsed pressure (intensity) beams were determined on the basis of preliminary measurements in water. First, the transducer excitation voltage, providing generation of the beam with the selected average acoustic power measured by the Ultrasound Power Meter, was determined. As mentioned above, the transducer was excited by 1000-cycle sinusoidal pulses generated from an arbitrary function generator Agilent 33250 and amplified by the power amplifier ENI3100LA. The source pressure amplitude and the initial intensity I_{SATA} of the generated beam for each voltage applied was determined by two methods: (1) using the measurements of the averaged radial pressure distribution near the transducer radiating surface using the calibrated 0.2 mm needle hydrophone S/N1661 (Precision Acoustics, Dorchester, UK) and (2) using the measurements of the average acoustic power using Ultrasound Power Meter UPM-DT-10AV (Ohmic Instruments Co., Easton, USA). The measurements by the needle hydrophone were carried out laterally at the axial distance of 1 mm from the transducer surface for the tone bursts with duration of 8 µs and PRF of 0.1 kHz. The RMS value for the pulse duration was recorded with a LeCroy 62xi oscilloscope. The sensitivity of the needle hydrophone for the frequency used was equal to 59.7 mV/MPa. The convergence of the source pressure amplitudes obtained by the two methods was within 4.4% and of the initial intensities or powers was within 9%.

The spatial acoustic pressure distributions in the ultrasonic beams used were measured in water under free field conditions using broadband bilaminar membrane PVDF hydrophone (with active electrode of 0.5 mm in diameter).

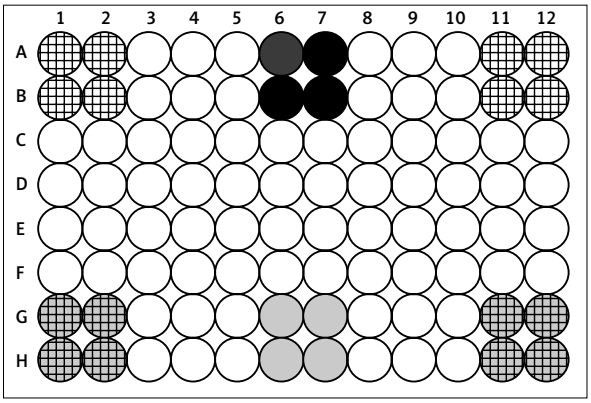


Fig. 2. Schematic arrangement of 4 groups of wells in each plate according to the kind of factor impacting cells: white – empty wells, black – control wells, gray – 5-aminolevulinic acid (ALA) alone wells, grid on a white background – ultrasound (US) alone wells, grid on a gray background – ALA + US wells.

Sonodynamic therapy protocols

The rat RG2 glioma cells were seeded on the bottom of 24 wells (6 x 4 wells) in 96-well polystyrene plates (Cellstar 96 Well Cell Culture Plate, Greiner Bio-One, USA), as shown in Figure 2.

200 µl of the cell suspension in DMEM medium supplemented with a 10% FBS, containing the same number of cells (2 x 10^4) were introduced to each well. After 24 h incubation at 37°C the medium was changed to DMEM without FBS or to DMEM without FBS but with ALA, and the cells were exposed for 3 min to ultrasound. During sonication a bottom of each plate was immersed in a water bath with temperature set to 21°C. The thickness of the bottom of polystyrene plates was about 1 mm.

The wells in each plate were allocated to 4 groups: 1) No exposure (Control); 2) Exposure to ALA; 3) Exposure to US and 4) Exposure to ALA + US. For ALA and ALA + US experiments the cells were incubated for 6 h in serum-free DMEM with 100 µM ALA to give them time to take up ALA and convert it to protoporphyrin IX. For the Control and US only experiments the same amount of DMEM was used. In the US and ALA + US experiments the cells were sonicated by pulsed ultrasound at a resonance frequency of 1 MHz and acoustic power varied from 2 W to 6 W (spatial-averaged temporal-averaged intensity [I_{SATA}] in the region of cells varied from 0.5 to 1.5 W/cm^2) during 3 min exposure. As demonstrated in the previous report [8] for such intensity levels and exposure time lethal thermal effects in the region of cells are not reached. After the treatment procedure the cells were re-suspended in fresh DMEM and subjected to further analyses.

Cell viability detection

To evaluate the effects of ALA and/or US on the rat RG2 glioma cells viability, a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used. Following exposure to ALA and/or US the cells were incubated at 37°C for 24 h, then 15 µl solution of MTT at a concentration of 5 mg/ml was added to 150 µl of culture medium in each of these wells (final concentration of MTT was 0.5 mg/ml). After 3 h incubation the media were removed. The formazan crystals were dissolved in 200 µl of dimethyl sulfoxide and this solution was added to each well. The absorbance of cells at 570 nm was measured using an Epoch micro-plate reader (Bio-Tek, ELX800, USA) in relation to the reference value at 630 nm. The viability of treated cells was determined by comparing to the untreated ones in the Control group.

Cell morphology and visualization of apoptosis

After the experiments and 24 h incubation the cells were stained by the Hoechst 33342 Nuclear Staining Dye (Invitrogen) with a concentration of 1 µg/ml at 37°C for 10 min. Nuclear Morphology of cell nuclei (chromatin condensation, presence of apoptotic bodies) was evaluated under an inverted fluorescence microscope IX81 Cell R equipped with a LUCPlanFLN objective (Olympus).

Statistical analysis

Statistical evaluation of data was performed using Graph-Pad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA, USA). One way ANOVA was followed by Bonferroni's multiple comparisons *post hoc* test. Differences were considered significant when $p < 0.05$ *vs* Control ($n = 8$). There were performed 2 independent MTT experiments with 8 repeats as well as 2 independent experiments of staining by Hoechst with 4 repeats.

Results

Enhancement of ultrasound induced cell killing during ALA-mediated SDT

As shown in Figure 3 at the exposure to US of 2 W cell survival for ALA + US group did not significantly differ from the respective controls (ALA alone), indicating no sonosensitization. However, when 6 W ultrasound was used, the cytotoxic effect in ALA + US group after 24 h was significant (see Fig. 4).

Changes in morphology of cell nuclei

The changes in cellular morphology, quantity of cells and chromatin condensation/apoptosis induced by ALA-mediated SDT on rat RG2 glioma cells depending on the acoustic power of ultrasound used are shown in Figures 5 and 6.

Discussion

In the present study, rat RG2 glioma cells were exposed to 5-aminolevulinic acid and afterwards son-

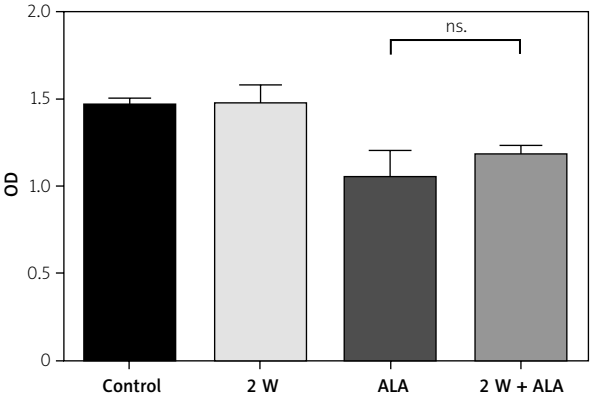


Fig. 3. Cytotoxic effect of 5-aminolevulinic acid and ultrasound (ALA + US) (2 W, 3 min. exposure) on viability of rat RG2 glioma cells after 24 h (mean ± SD, $n = 8$).

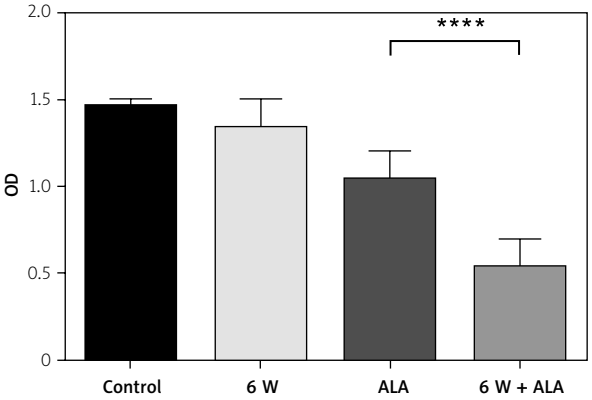


Fig. 4. Cytotoxic effect of ultrasound 5-aminolevulinic acid and ultrasound (ALA + US) (6 W, 3 min. exposure) on viability of rat RG2 glioma cells after 24 h (mean ± SD, *n* = 8).

icated with pulsed low intensity ultrasound waves. Our aim was to look for the sonodynamic effect, and in particular for signs of SDT-mediated apoptotic cell death. As mentioned in the introduction, the RG2 cells seem to be better suited for preclinical research using orthotopic implantation than the C6 cells because they are less immunogenic and their growth in brain is more infiltrative. Nevertheless, the RG2 glioma cells are much less used in research than the C6 glioma cells. In particular, whereas several papers described reactions of the C6 cells in vivo to ALA-mediated PDT [2,7,17] and SDT [8,9,14], we were unable to find any report on experiments with the RG2 cells and either SDT or PDT.

Currently the mechanism of sonosensitization of glioma cells by ALA is poorly understood. Results obtained in the present study provide evidence for ALA-mediated sonosensitization of RG2 rat glioma cells *in vitro*, but this effect was evident only when the cells pre-incubated with ALA were sonicated by US with intensity of 6 W, which we consider the upper limit of US dose that is not directly lethal to the cells due to rise in temperature. In the present experiments, neither the concentration of PpIX, nor the amount of free radicals in the cells were measured, therefore no direct evidence can be presented for a mechanism of sonosensitization. While other mechanisms of cytotoxicity such as necrosis or autophagy cannot be excluded, the present experiments provided evidence of apoptosis being the major event evoked by sonosensitization of RG2 cells with ALA.

The SDT technique is tested not only *in vitro*, but also *in vivo*. A selective effect of low intensity US against C6 glioma cells implanted to the rat brain was achieved for the first time with the use of Rose Bengal dye given intravenously as a sonosensitizer [10]. Since then two other publications have reported on experiments in which ALA-mediated STD were tested *in vivo* on orthotopically transplanted C6 glioma [5,11]. In

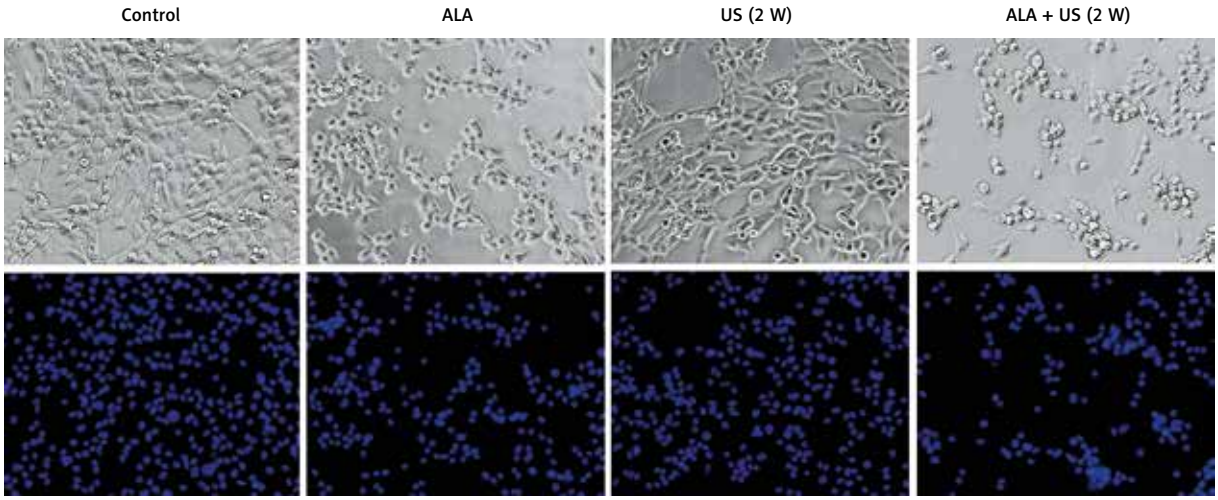


Fig. 5. The morphology, quantity and chromatin condensation/apoptosis of RG2 rat glioma cells 24 h after 5-aminolevulinic acid (ALA)-mediated SDT (3-minute exposure to 2 W ultrasound). The top images show cell cultures visualized by a light microscope (contrast phase). The bottom images show the nuclear morphology (Hoechst 33352 staining, fluorescence microscopy). There are slight differences in the cell number and their morphological features between ALA, ultrasound (US) (2 W) and ALA + US (2 W) groups.

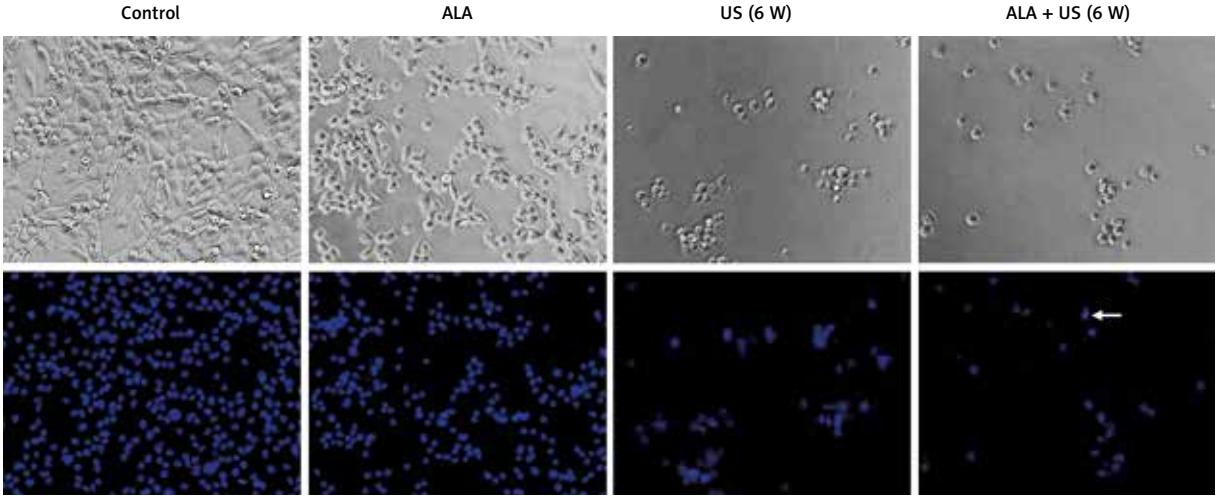


Fig. 6. The morphology, quantity and chromatin condensation/apoptosis of RG2 rat glioma cells 24 h after 5-aminolevulinic acid (ALA)-mediated SDT (3-minute exposure to 6 W ultrasound). The top images show cell cultures visualized by a light microscope (contrast phase). The bottom images show the nuclear morphology (Hoechst 33352 staining, fluorescence microscopy). There are noticeable differences in the cell number and their morphological changes between ALA alone, ultrasound (US) (6 W) alone and ALA + US (6 W) groups. Arrows point to nuclei with chromatin condensation, arrowheads show apoptotic bodies.

these papers SDT-induced decreases in gross tumor volume evaluated post-mortem were shown.

Effective therapy of human malignant gliomas would require elimination of clonogenic glioma cells which spread beyond tumor margins that are visible during surgery or visualized by radiological techniques [6]. These cells, some of which are located at a considerable distance from gross tumor margins, cause glioma recurrence. While it has already been shown that ALA-mediated SDT applied to intracranially growing C6 glioma in rat can cause gross tumor margins to shrink, it remains to be investigated whether this technique can also eliminate clonogenic glioma cells located at a distance from the tumor. For such study RG2 glioma cell line would certainly be a better choice.

Disclosure

Authors report no conflict of interest.

References

1. Barth RF, Kaur B. Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. J Neurooncol 2009; 94: 299-312.
2. Beck TJ, Burkanas M, Bagdonas S, Krivickiene Z, Beyer W, Sroka R, Baumgartner, Rotomskis R. Two-photon photodynamic therapy of C6 cells by means of 5-aminolevulinic acid induced protoporphyrin IX. J Photochem Photobiol B 2007; 87: 174-182.
3. Cuddapah VA, Robel S, Watkins S, Sontheimer H. A neurocentric perspective on glioma invasion. Nat Rev Neurosci 2014; 15: 455-465.
4. Grieb P. 5-Aminolevulinic acid (ALA) and its applications in neurosurgery. Neurol Neurochir Pol 2004; 38: 201-207.
5. Jeong EJ, Seo SJ, Ahn YJ, Choi KH, Kim KH, Kim JK. Sonodynamically induced antitumor effects of 5-aminolevulinic acid and fractionated ultrasound irradiation in an orthotopic rat glioma model. Ultrasound Med Biol 2012; 38: 2143-2150.
6. Ji M, Lewis S, Camelo-Piragua S, Ramkissoon SH, Snuderl M, Venneti S, Fisher-Hubbard A, Garrard M, Fu D, Wang AC, Heth JA, Maher CO, Sanai N, Johnson TD, Freudiger CW, Sagher O, Xie XS, Orringer DA. Detection of human brain tumor infiltration with quantitative stimulated Raman scattering microscopy. Sci Transl Med 2015; 7: 309ra163.
7. Kamoshima Y, Terasaka S, Kuroda S, Iwasaki Y. Morphological and histological changes of glioma cells immediately after 5-aminolevulinic acid mediated photodynamic therapy. Neurol Res 2011; 33: 739-746.
8. Kujawska T, Secomski W, Bilmin K, Nowicki A, Grieb P. Impact of thermal effects induced by ultrasound on viability of rat C6 glioma cells. Ultrasonics 2014; 54: 1366-1372.
9. Li JH, Chen ZQ, Huang Z, Zhan Q, Ren FB, Liu JY, Yue W, Wang Z. In vitro study of low intensity ultrasound combined with different doses of PDT: Effects on C6 glioma cells. Oncol Lett 2013; 5: 702-706.
10. Nonaka M, Yamamoto M, Yoshino S, Umemura S, Sasaki K, Fukushima T. Sonodynamic therapy consisting of focused ultrasound and a photosensitizer causes a selective antitumor effect in a rat intracranial glioma model. Anticancer Res 2009; 29: 943-950.
11. Ohmura T, Fukushima T, Shibaguchi H, Yoshizawa S, Inoue T, Kuroki M, Sasaki K, Umemura S. Sonodynamic therapy with

5-aminolevulinic acid and focused ultrasound for deep-seated intracranial glioma in rat. Anticancer Res 2011; 31: 2527-2533.
12. Shibaguchi H, Tsuru H, Kuroki M, Kuroki M. Sonodynamic cancer therapy: a non-invasive and repeatable approach using low-intensity ultrasound with a sonosensitizer. Anticancer Res 2011; 31: 2425-2429.
13. Tetard MC, Vermandel M, Mordon S, Lejeune JP, Reyns N. Experimental use of photodynamic therapy in high grade gliomas: a review focused on 5-aminolevulinic acid. Photodiagnosis Photodyn Ther 2014; 11: 319-330.
14. Tserkovsky DA, Alexandrova EN, Chalau VN, Istomin YP. Effects of combined sonodynamic and photodynamic therapies with photolon on a glioma C6 tumor model. Exp Oncol 2012; 34: 332-335.
15. Weizsäcker M, Nagamune A, Winkelströter R, Vieten H, Wechsler W. Radiation and drug response of the rat glioma RG2. Eur J Cancer Clin Oncol 1982; 18: 891-895.
16. Wood AK, Sehgal CM. A review of low-intensity ultrasound for cancer therapy. Ultrasound Med Biol 2015; 41: 905-928.
17. Wu SM, Ren QG, Zhou MO, Peng Q, Chen JY. Protoporphyrin IX production and its photodynamic effects on glioma cells, neuroblastoma cells and normal cerebellar granule cells in vitro with 5-aminolevulinic acid and its hexylester. Cancer Lett 2003; 200: 123-131.

Original paper

Folia Neuropathologica

Characteristics of the expression of KAI1/CD82 and PDGFRβ and their impact on glioma progression

Michal Paradowski[1], Malgorzata Bilinska[1], Julia Bar[2]

[1]Department of Neurology, Wroclaw Medical University, [2]Department of Immunopathology and Molecular Biology, Wroclaw Medical University, Wroclaw, Poland

Folia Neuropathol 2016; 54 (3): 241-248 DOI: 10.5114/fn.2016.62554

Abstract

The biological features of glioma cells may define their clinical outcome. Little is known about the interactions between KAI1/CD82 metastatic suppressor protein and PDGFRβ in gliomas. The aim of the study was to examine KAI1/CD82 and PDGFRβ expression in gliomas in order to find the impact of these proteins on progression of the tumors. PDGFRβ, KAI1/CD82 protein expression and mRNA of genes were evaluated on eighty four paraffin-embedded tissue of gliomas using immunohistochemical staining and RT-PCR analysis. The PDGFRβ expression was higher in IV/III than in I/II glioma grades (p = 0.0004). The level of mRNA PDGFRβ was associated with the degree of PDGFRβ immunoreactivity. Downregulation of KAI1/CD82 was associated with tumor malignancy (p = 0.007). The increased level of KAI1/CD82 gene expression (3-4-fold) was found in gliomas with strong KAI1/CD82 immunoreactivity. The parallel KAI1/CD82 and PDGFRβ expression was more significantly associated with cases in a group graded as III and IV than in a group graded as I/II (p = 0.002).

We found that a loss of KAI1/CD82 and an increase in PDGFRβ expression in gliomas relate to a progressive tumor growth. The correlation between PDGFRβ and KAI1 expression in high grade gliomas suggests that a direct or indirect interaction between these proteins might have an impact on cell motility and invasive behavior of the tumor.

Key words: *gliomas, KAI1/CD82, PDGFRβ, immunohistochemistry, RT-PCR.*

Introduction

Gliomas are the most frequent primary neoplasms of the central nervous system (CNS). Most of them are characterized by their extensive invasion into the brain parenchyma [17,19,20]. Grade I/II astrocytomas are slow-growing tumors without aggressive features, whereas grade III and IV gliomas possess a malignant phenotype associated with high proliferative activity and vascular formation [18,19]. Glioblastoma (grade IV) is one of the most aggressive and deadly malignant brain tumors with an average survival time of 15 months after diagnosis [8,13,18,20]. Most primary glioblastomas develop *de novo* but some parts of diffuse astrocytomas grade II and III may progress to grade IV as secondary glioblastomas [12,25].

The high infiltration capacity of individual glioma cells is related to the unique biological features of these cells [5,12,18]. According to some authors, the migratory behavior of glioma cells observed during the tumor progression might be a result of the activa-

Communicating author

Julia Bar, Department of Immunopathology and Molecular Biology, Wroclaw Medical University, 213 Borowska St., 50-368 Wroclaw, Poland, phone: +48 71 784 00 32, e-mail: julia.bar@umed.wroc.pl

tion of surface receptors and signaling pathways [19]. A number of alternations in different genes have been identified in human gliomas and some of them are involved in glioma progression [8]. Among the growth factors, the platelet-derived growth factor (PDGF) has been well described in glioblastomas [19,20].

A lot of data suggested that the PDGF receptor activates a number of downstream signal transduction pathways including PI3K/Akt/mTOR and Ras/Raf/MAPK pathways and may play an important role in both normal development and tumorigenesis of the CNS [19,20]. Overexpression of PDGF ligands and receptors are frequent events in human gliomas regardless of the tumor grade. Their expression pattern in gliomas suggests the presence of autocrine and paracrine stimulatory loops [20]. It was revealed that PDGF signaling alone may be sufficient to induce glioma formation [10]. Blocking of PDGF signaling strongly implies that PDGF signaling is necessary and sufficient to maintain the malignant phenotype in the *in vitro* model [31].

The specific biological mechanisms that mediate tumors' invasive nature are still unknown [30]. Glioma progression may be compared to other solid tumor metastasis process. This process involves a molecular mechanism which requires the contribution of a multiple gene alteration [15,30]. The role of metastasis suppressor genes has been discussed very rarely in gliomas [8,19]. The most interesting is the KAI1/CD82 gene originally identified as a putative metastasis suppressor gene for prostate cancer [9]. The KAI1/CD82 gene is a member of the tetraspan transmembrane super family (TM4SF) [30]. KAI1/CD82 is expressed in various human tissues and plays an important role in cell fusion, adhesion, migration, signaling, fertilization, differentiation and invasion [11]. Downregulation of both KAI1/CD82 mRNA and protein expression is observed during progression and increased invasive behavior of tumors [7,15,30]. Several mechanisms have been proposed by which KAI1/CD82 might influence and control tumor cell behavior [29]. It was established that KAI1/CD82 plays an important role in regulating melanoma cell migration through the controlling of Rho and GTPases signaling activity [23]. Another study revealed that KAI1/CD82 might have an inhibitory role in the PI3K/AKT pathway [1,6,23]. So far, the correlation between KAI1/CD82 expression and the signal transduction pathway has been examined in breast and ovarian cancers [29].

Little is known about the interaction between the members of metastasis suppressor genes and kinase receptors in primary brain tumors and their role in glioma cell behavior. The relationship between KAI1/CD82 and tyrosine kinase receptor PDGFRβ has not been evaluated in gliomas. To investigate the possible suppressive role of KAI1/CD82 in gliomas and their influence on PDGFRβ expression we examined KAI1/CD82 and PDGFRβ expression and the relation between them in gliomas in order to find the impact of these proteins on progression of gliomas.

Material and methods

The study was performed on tissue sections from 84 patients diagnosed with primary gliomas hospitalized in the Clinic of Neurosurgery of the Wroclaw Medical University, Poland between 2007 and 2012. Tumor tissues were obtained at initial surgery. None of the patients received any treatment before the operation. All tumors were histologically verified to confirm the diagnosis, histological type and tumor grade according to established criteria classification of the central nervous system tumors by the World Health Organization (WHO) [17]. Based on the WHO classification, gliomas were subdivided into the following groups: grade I – 6 cases (6 cases of pilocytic astrocytoma), grade II – 24 cases (22 cases of fibrillary astrocytoma, 2 cases of oligodendroglioma), grade III – 15 cases (12 cases of anaplastic astrocytoma, 3 cases of anaplastic oligodendroglioma) and grade IV – 39 cases of glioblastoma multiforme.

The study was conducted in accordance with the declaration of Helsinki. This study was approved by the Local Ethic Committee of Human Research of the Medical University of Wroclaw, Poland (permission no. 37/2012). Written informed consent was obtained from all participants.

Immunohistochemical staining

Immunohistochemical staining (IHC) for the analyzed proteins was performed on paraffin-embedded tissue using the Universal DakoCytomation LSAB + Kit, Peroxidase procedure (LSAB+ Kit:HRP, Dako, Copenhagen, Denmark) and the following primary monoclonal antibodies: anti-KAI1 (G2) (Santa Cruz Biotechnology, USA) and anti PDGFRβ (28E1) (Cell Signaling Technology, USA).

Five-micrometer sections from one selected block from each lesion were deparaffinized and boiled for

3 x 5 minutes for each antibody in citrate buffer (pH 6.0) at 700 W in a microwave oven. After the microwave treatment, the tissue sections were slowly cooled for 20 minutes. Endogenous peroxidase reactivity was blocked with 3% H_2O_2 and nonspecific tissue reactions with 10% BSA (bovine serum albumin). Tissue specimens were incubated with primary antibodies (anti-KAI1 anti-PDGFRβ) overnight at 4°C. Following washing with 0.1 M Tris-buffer, pH = 7.4 (TBS), the tissue specimens were incubated with a secondary biotinylated rabbit antibody, anti-mouse IgG (Dako, Copenhagen, Denmark) and with streptavidin-horseradish peroxidase-conjugated (Dako) both for 15 minutes at room temperature. After washing with TBS, the antigen-antibody reaction was visualized by DAB (3,3'-diaminobenzidine) (Dako, Denmark) as a chromogen (8 minutes, room temperature). Sections were counterstained with hematoxylin and mounted. The incubation buffer (TBS) without the primary antibody was used as a negative control. The internal positive controls were performed according to the manufacturer's protocol.

The preparations were evaluated under a BX-51 Olympus light microscope. The localizations, distributions and intensity of immunostaining were evaluated in the tissue sections. For KAI1 and PDGFRβ membrane immunostaining was considered as positive when at least 10% of tumor cells were stained.

The intensity of staining was scored as 0 for negative, + weak, ++ moderate, and +++ strong. The immunohistochemical analyses were interpreted without prior knowledge of the clinical information.

Reverse transcription polymerase chain reaction

For reverse transcription polymerase chain reaction (RT-PCR) analysis of KAI1/CD82 and PDGFRβ genes, immunopositive cases for both proteins were divided into three groups: 10-40% positive tissue, 41-70% and 71-100% positive tissue. Each group consisted of 10 cases.

We used the method of RT-PCR for analysis of KAI1/CD82 and PDGFR gene expression. RNA was isolated from the freezing tissue of 30 patients with gliomas (which revealed a different level of KAI1/CD82 and PDGFRβ expression in IHC staining) according to the method of RNeasy Plus Mini from Qiagen. The reverse transcription was performed with QuantiTect Reverse Transcription kit (Qiagen). The estimation of KAI1/CD82 and PDGFRβ gene expression was performed in Rotor-Gene TM. The reaction mixture for RT-PCR volume of 25 µl contained 2 µl complementary DNA (cDNA) and 2 µl gene sequences of primers for KAI1/CD82 (Hs_CD82_1_SG Quant Tect Primer Assay Qiagen), PDGFRβ (Forward – 5'-AATGTCTCCAGCACCTTCGT-3'489-509, Reverse – 3'-AGCGGATGTGGTAAGGCAATA-5')(1177-1156)andreagents Rotor-Gene SYBR Green Master Mix and RNase-free water. The standard curve plotted on the basis of the reference gene peptidyl prolyl isomerase C (cyclophilin C, CYCC, Hs_PPIA Quant Tect Primer Assay, Qiagen) with a different concentration; diluted 10-, 100- and 1000-fold. The RT-PCR reaction consisted of one cycle PCR initial activation step of 95°C for 5 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C of 10 seconds.

Statistical analysis

Correlations between KAI1 and PDGFRβ expression and glioma grade malignancy were statistically studied by χ^2 test. Associations between KAI1 and PDGFRβ expression were analyzed by Spearman's rank correlation. Differences were considered as significant when $p \leq 0.05$.

Results

PDGFRβ expression and analysis of mRNA PDGFRβ levels in gliomas

Membrane PDGFRβ expression was found in 44/84 (52.3%) gliomas. The majority of cases revealed PDGFRβ immunopositivity in 10-40% of tumor tissue. In the vast majority of gliomas, the immunoreactivity for PDGFRβ was observed in the group of cells distributed in different parts of tumor tissue (Fig. 1). The strong reactivity for PDGFRβ (70-100% positive tissue) was found only in 9.09% of cases. In glioblastomas PDGFRβ expression was stronger than in fibrillary astrocytomas and oligodendrogliomas ($p = 0.0001$). The PDGFRβ expression revealed differences in IV/III tumor grades versus I/II glioma grades ($p = 0.0004$) (Fig. 2). Similar differences were found for the mean value for PDGFRβ expression between low grade gliomas (I and II) [GI 1.666 $\pm$ 4.082 (SD), GII 6.25 $\pm$ 19.067 (SD)] compared with high grade gliomas (III and IV) [GIII 20.666 $\pm$ 22.834 (SD), GIV 31.794 $\pm$ 28.457 (SD)], $p = 0.001$. In gliomas showing PDGFRβ immunoreactivity of 70-100% of tumor tissue, the level of mRNA PDGFRβ was higher (increased 3-5-fold) compared to

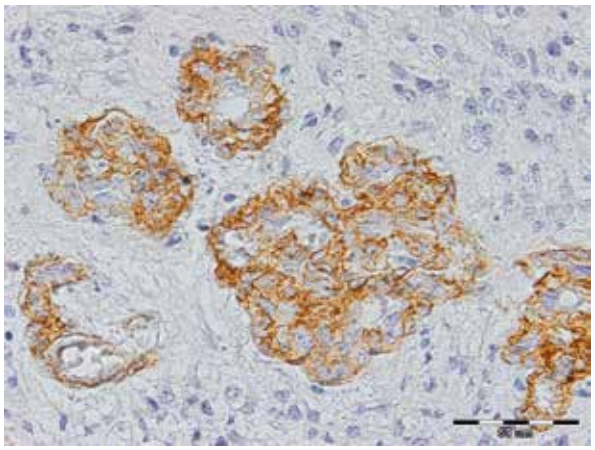


Fig. 1. In glioblastoma tissue, PDGFRβ is strongly expressed in cell membrane (avidin-biotin staining × 200).

cases with PDGFRβ immunoreactivity of 40-70% of positive cases (increased 1.5-2 fold).

KAI1/CD82 expression and analysis of mRNA KAI1/CD82 levels in gliomas

KAI1/CD82 immunostaining was observed on the membrane and in the cytoplasm of tumor cells in 75.0% of gliomas. The membrane staining dominated and was found in a different range of tumor cells (10-100% tumor tissue) in individual gliomas. Only 17.4% of cases showed strong immunoreactivity for KAI1/CD82 ranging between 70% and 100% positive tissue (Fig. 3). Statistical differences were observed

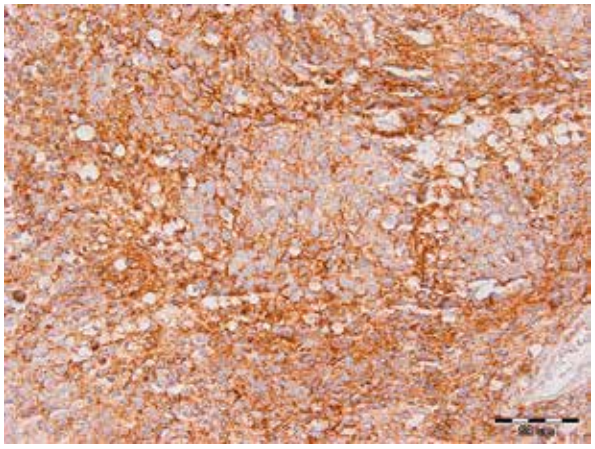


Fig. 3. KAI1/CD82 expression in gliomas graded as GII. Glioma tissues showed a strong membrane and cytoplasm expression of KAI/1CD82 protein (avidin-biotin staining × 200).

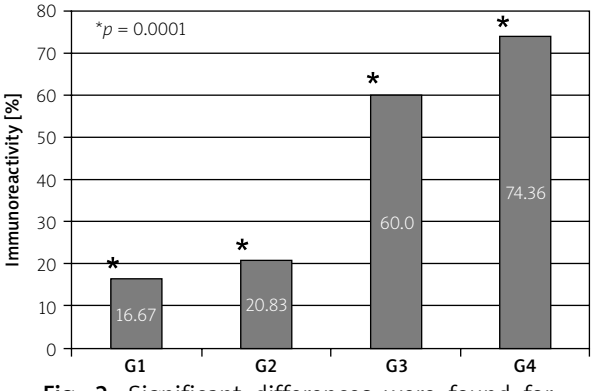


Fig. 2. Significant differences were found for PDGFRβ expression between gliomas graded as IV/III than in I/II grades (p = 0.0004).

for KAI1/CD82 expression between GI/II vs. GIII/IV (p = 0.01) (Fig. 4). Similarly, the mean value of KAI1/CD82 expression in I/II [GI 58.333 ± 23.1666 (SD), GII 52.916 ± 32.900 (SD)] and III/IV [GIII 30.666 ± 28.652 (SD), GIV 25.128 ± 27.991 (SD)] tumor grades showed significant differences (p = 0.001). Comparing the extent of KAI1 immunoreactivity in relation to tumor grades, a significant downregulation of KAI1/CD82 protein expression in III/IV compared with I/II tumor grades was found (p = 0.007) (Fig. 5). The increased level of KAI1/CD82 gene expression (3-4-fold) was observed in gliomas with immunoreactivity for KAI1/CD82 above 50% of tumor tissue with strong intensity of staining defined as +++.

No correlation between KAI1/CD82 and PDGFRβ expression was observed in the whole group of gliomas ($p \leq 0.05$). Moreover, the parallel KAI1/CD82 and PDGFRβ expression was observed more frequently in a group graded as III and IV than in a group graded

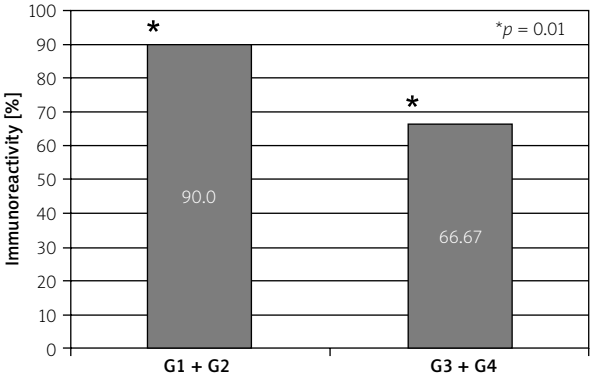


Fig. 4. The mean value of KAI1/CD82 expression in I/II and III/IV glioma grades showed significant differences (p = 0.001).

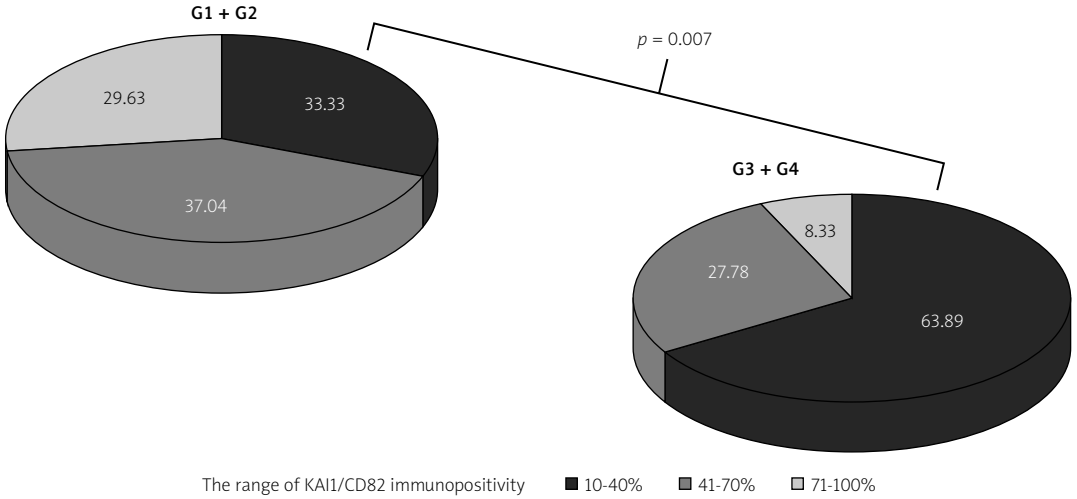


Fig. 5. The extent of KAI1 immunoreactivity related to glioma grades. Significant downregulation of KAI1/CD82 protein expression was found between gliomas graded as III/IV compared with I/II tumor grades (p = 0.007).

as I/II. Observed differences were statistically significant (p = 0.002) (Fig. 6).

Discussion

The common feature of malignant brain tumors is local invasiveness to the surrounding tissue. Specific chemotactic signaling pathways are involved in the regulation of tumor cell motility [22]. In agreement with earlier data [14,16,21,31], we found the PDGFRβ expression more frequently in grade III and IV than in grade I and II gliomas. Some authors suggest that the autocrine and paracrine stimulation of PDGFRβ could play an essential role in glial tumorigenesis [4,31]. It was revealed that during glioma progression the PDGFRβ stimulation can induce the activation of different signaling receptors like EGFR, Notch which lead to dedifferentiation of glioma cells, and increase cell motility and malignancy of the tumor [8,31].

The correlation of PDGFRβ expression with the WHO high grade gliomas observed in the current study is partly linked to other data which suggest that this receptor is a crucial factor which regulates angiogenesis and might indirectly facilitate dissemination of tumor cells from primary tumor mass and invade normal brain tissue [21]. The role of PDGFRβ in invasion of tumor cells was revealed by inhibition of cell migration by suppression of PDGFRβ tyrosine phosphorylation [16]. Based on previous reports and our data we postulate that a high PDGFRβ expression in low grade gliomas might characterize the subset of gliomas with biological aggressive behavior and suggest that depending on the PDGFRβ signaling pathway, activation is possible in an early oncogenic event in gliomas [14]. Similarly to other tumors such as breast or liver tumors, a high PDGFRβ expression in gliomas might contribute to the decreased cell-cell adhesion and promote metastatic capacity of tumor cells [22]. Some data indicate that PDGFRβ overexpression is associated with epithelial-to-mesenchymal transition (EMT) [22]. Moreover, the PDGFRβ expression following EMT transient state may induce the metastatic process [27]. Additionally, in this group

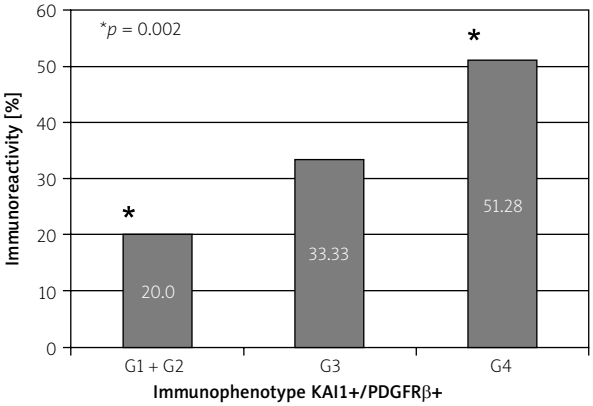


Fig. 6. The parallel KAI1/CD82 and PDGFRβ expression dominated in gliomas graded as III and IV than in gliomas graded as I/II. Observed differences were statistically significant (p = 0.002).

of tumors a high expression of chitinase-like protein (YKL40) was found [2]. The authors suggested that YKL40 positivity contributes to progression of glioblastoma [2]. In gliomas with a high tumor grade the EMT transient state is often observed and in these tumors both mRNA PDGFRβ and its protein are highly expressed [18]. Authors claim that glioblastoma cells which have mesenchymal features and possess a high PDGFRβ expression indicate that this biological feature might characterize the group of tumors with increased cell plasticity [14]. This observation might be compared with our previous study which showed that the E-cadherin expression was weaker or absent in high than low grade gliomas [3]. In the current study, the PDGFRβ expression was analyzed in the same set of gliomas and obtained data related to the E-cadherin expression (unpublished data).

There are no data describing the role of KAI1/CD82 expression in gliomas by other authors. In the present study we analyzed the mRNA KAI1/CD82 gene and its protein gliomas in order to show the impact of KAI1/CD82 expression on glioma growth. Some authors found that loss of the KAI1/CD82 protein expression is associated with the invasive growth of tumors and metastasis [7,15]. In gliomas the invasive growth is associated with the migration of tumor cells to the surrounding tissue, so the question is whether KAI1/CD82 may suppress the migratory function of tumor cells in gliomas similarly to colorectal carcinoma [27].

The current study found a high KAI1/CD82 expression in low and loss in high grade gliomas. These results are consistent with previous data that KAI1/CD82 expression is associated with increased invasive behavior of solid tumors [7,26,29]. In analyzed glioma specimens overexpression of KAI1/CD82 was observed in glioma cells without malignancy features regardless of the tumor grade whereas the majority of malignant cells were negative for KAI1/CD82 immunostaining. The different pattern of KAI1/CD82 expression observed in this study might reflect the high degree of intratumoral heterogeneity of the primary tumor that progression and metastasis are spawned by selective subclone of cells [28]. Recently, data revealed that upregulation of microRNA-210 induced cell proliferation and migration of glioblastoma cells [32]. We postulate that KAI1/CD82 protein expression in gliomas might play a similar role to that in tumors with metastasis to other organs [7,23]. Firstly, KAI1/CD82 as a metastasis suppressor protein might inhibit the migratory ability of glioma cells, so we suggest that a high expression of KAI1/CD82 in low grade gliomas might reduce the risk of secondary glioblastoma development. Secondly, the other function of KAI1/CD82 protein could be considered in gliomas like regulation by microRNA. An experimental study has shown that KAI1/CD82 is able to inhibit the signaling PI3K/AKT pathway in breast, bladder, and melanoma cancer cell lines [23,24]. Taking into account that the PI3K/AKT pathway is often activated by PDGFR receptors during glioma progression, we suggest that the suppression of the PI3K/AKT pathway by KAI1/CD82 protein might limit the proliferation and spread of glioma cells [19,23].

The present study is the first one to investigate the association between KAI1/CD82 protein and PDGFRβ expression in gliomas. Interestingly, we found that KAI1/CD82 expression is closely related to PDGFRβ expression in high grade glioma malignancy. Our results demonstrate that parallel expression of both biomarkers in glioblastomas might identify the cases where KAI1/CD82 might lead to a reduction in PDGFRβ activity and probably inhibit angiogenesis dependent on PDGFRβ expression. Our observations are partly consistent with the studies reporting that the KAI1/CD82 attenuated pathway depends of receptor tyrosine kinase (RTKs) activity [7,24,29]. Based on earlier reports which describe the association between KAI1/CD82 and surface receptor responsible for a different signaling pathway we suggest that such mechanism may occur in gliomas [31].

Conclusions

We found that a loss of KAI1/CD82 and an increase in PDGFRβ expression in gliomas relate to a progressive tumor growth. A correlation between PDGFRβ and KAI1/CD82 expression in high grade gliomas suggests that a direct or indirect interaction between these proteins might influence the cell motility and invasive behavior of the tumor.

Acknowledgments

This work was supported by Wroclaw Medical University grant Pbmn 113.

Disclosure

Authors report no conflict of interest.

References

1. Badyopadhyay S, Zhan R, Chaudhuri A, Watabe M, Pai SK, Hirota Sh, Hosobe S, Tsukada T, Miura K, Takano S, Wang Y, Mohinta S, Saito K, Pauza ME, Hayashi S, Wang Y, Mohinta S, Mashimo T, Iiizumi M, Furuta E, Watabe K. Interaction of KAI1 on tumor cells with DARC on vascular endothelial leads to metastasis suppression. Nat Med 2006; 12: 933-938.
2. Batista KMP, Vega IF, de Eulate-Beramendi SA, Morales JCG, Kurbanov A, Asnel D, Meilan A, Astudillo A. Prognostic significance of the markers IDH1 and YKL40 related to the subventricular zone. Folia Neuropathol 2015; 1: 52-59.
3. Bar JK, Zub L, Lis-Nawara A, Noga L, Jelen M, Paradowski B. Expression and interaction between cell adhesion molecules CD44v6 and E-cadherin in human gliomas. Adv Clin Exp Med 2014; 5: 827-834.
4. Barrett A, Evans IM, Frolov A, Britton G, Pellet-Many C, Yamaji M, Mehta V, Bandopadhyaya R, Ningning L, Brander S, Zachary JC, Frankel P. Critical role for DOK1 in PDGF-BB stimulated glioma cell invasion via p130Cas and Rap1 signalling. J Cell Sci 2014; 127: 2647-2658.
5. Chen H, Shah AS, Reda EG, Grossman SA. Transmission of glioblastoma multiforme after bilateral lung transplantation. J Clin Oncol 2008; 26: 3284-3285.
6. Choi UJ, Jee BK, Lim Y, Lee K-H. KAI1/CD82 decreases Rac1 expression and cell proliferation through PI3K/Akt/mTOR pathway in H1299 lung carcinoma cells. Cell Biochem Function 2009; 27: 40-47.
7. Dai W, Wang C, Wang F, Wang Y, Shen M, Chen K, Cheng P, Zhang Y, Yang J, Zhu R, Zhang H, Li J, Zheng Y, Lu J, Zhou Y, Xu L, Guo Ch. Anti-miR-197 inhibits migration in HCC cells by targeting KAI 1/CD82. Biochem Bioph Res Commun 2014; 446: 541-548.
8. Dimov I, Tasić-Dimov D, Conic I, Stefanovic V. Glioblastoma multiforme stem cells. Sci World J 2011; 11: 930-958.
9. Dong TJ, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT, Barrett JC. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11.p11.2. Science 1995; 268: 884-886.
10. Ellis JA, Canoll P, McCormick PC, Feldstein NA, Anderson RC, Angevine PD, Kaiser MG, McCornik PC, Bruce JN, Ogden AT. Platelet-derived growth factor receptor (PDGFR) expression in primary spinal cord gliomas. J Neurooncol 2012; 106; DOI: 10.1007/s11060-011-0666-6.
11. Malik FA, Sandres AJ, Jiang WG. KAI-1/CD82, the molecule and clinical implication in cancer and cancer metastasis. Histol Histopathol 2009; 24: 519-530.
12. Fertetta M, Uhrbon L, Olofsson T, Pontén F, Westermak B. Sox10 has a broad expression pattern in gliomas and enhances platelet-derived growth factor-B – induced gliomagenesis. Mol Cancer Res 2007; 5: 891-897.
13. Kim ChS, Jung S, Jung TY, Jang WY, Sun HS, Ryu HH. Characterization of invading glioma cells using molecular analysis of leading-edge tissue. J Korean Neurosurg Soc 2011; 50: 157-165.
14. Kim Y, Kim E, Wu Q, Guryanova O, Hitomi M, Lathia JD, Serwanski D, Sloan A, Weil RJ, Lee J, NIshiyama A, Bao Sh, Hjelmeland AB, Rich JN. Rich platelet-derived growth factor receptors differentially inform intertumoral and intratumoral heterogeneity. Genes Development 2012; 16: 1247-1262.
15. Liu X, Guo XZ, Li HY, Chen J, Ren LN, Wu ChY. KAI1 inhibits lymphangiogenesis and lymphatic metastasis of pancreatic cancer in vivo. Hepatobiliary Pancreat Dis Int 2014; 13: 87-92.
16. Liu Y, Le W, Lin Y, Cheang TY, Wang M, Zhang H, Zhang L, Wang S. Gambogic acid induces G0/G1 cell cycle arrest and cell migration inhibition via suppressing PGFR receptor β tyrosine phosphorylation and Rac1 activity in rat aortic smouth muscle cells. J Atheroscler Thromb 2010; 17: 901-913.
17. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. WHO classification of tumours of the central nervous system. 4th ed. International Agency for Research on Cancer (IARC), Lyon 2007; 14-33.
18. Nager M, Bhardwaj D, Canti C, Medina L, Nogues P, Herreros J. β-catenin signaling in glioblastoma multiforme and glioma-initiating cells. Chem Res Prac 2012; DOI: 10.1155/2012/192362.
19. Nakada M, Kita D, Watanabe T, Hayashi Y, Teng L, Pyko IV, Hamada JI. Aberrant signaling pathways in glioma. Cancer 2011; 3: 3242-3278.
20. Nazarenko I, Hede SM, He X, Hedren A, Thompson J, Lindstrom MS, Nister M. PDGF and PDGR receptors in glioma. Upsala J Med Sci 2012; 117: 99-112.
21. Pfister C, Pfrommer H, Tatagiha MS, Roser F. Vascular endothelial growth factor signals through platelet-derived growth factor receptor β in meningiomas in vitro. Br J Cancer 2012; 107: 1702-1713.
22. Steller EJA, Raats DA, Koster J, Rutten B, Govaert KM, Emmik BL, Snoeren N, van Hooff SR, Holstege F, Maas C, Rinkes HM, Kranenburg O. PDGFRB promotes liver metastasis formation of mesenchymal-like colorectal tumor cells. Neoplasia 2013; 15: 204-217.
23. Tang T, Cheng Y, Martinka M, Ong CH, Li G. Prognostic significance of KAI1/CD82 in human melanoma and its role in cell migration and invasion through the regulation of ING4. Carcinogenesis 2014; 1: 86-95.
24. Tsai YCH, Weissman AM. Dissection the diverse function of the metastasis suppressor CD82/KAI1. FEBS Lett 2011; 585: 3166-3173.
25. Wan L, Pantel K, Kang Y. Tumor metastasis: moving new biological insights into the clinic. Nature Med 2013; 11: 1450-1464.
26. Wang G, Jiang H, Xu H, Sun Q, Zhou Y, Xiang P, Cheng Z, Zhang Y, Zhou Y, Guo Q, Du X, Xu S, Ma S, Chen Z. Clinical significance of KAI1/CD82 protein expression in nasopharyngeal carcinoma. Oncol Lett 2015; 9: 1681-1686.
27. Wu Q, Yang Y, Wu SH, Li W, Zhang NA, Dong X, Ou Y. Evaluation of the correlation of KAI/CD82, Cd44, MMP7, β-catenin in the prediction of prognosis and metastasis in colorectal carcinoma. Dig Pathol 2015; 10: 176-182.
28. Wu X, Northcott PA, Dubuc A. Clonal selection drives genetic divergence of metastatic medulloblastoma. Nature 2012; 482: 529-533.
29. You JJ, Hon B, Madigan MC, Rowe A, Sajinovic M, Russell PJ, Jackson P. An inverse relationship between KAI1 expression, invasive ability, and MMP-2 expression and activity in bladder cancer cell lines. Urol Oncol 2012; 30: 502-508.

30. Yu L, Zhou L, Wu Sh, Gong X, Feng Zh, Ma L, Zhu B, Yao N, Wang D, Dong H. Clinicopathological significance of cancer stem cells marked by CD133 and KAI1/CD82 expression in laryngeal squamous cell carcinoma. World J Surg Oncol 2014; 12: 118.
31. Zhang J, Chen T, Mao Q, Lin J, Jia J, Li Sh, Xiong W, Lin Y, Liu Zh, Liu X, Zhao H, Wang G, Zheng D, Qiu Sh, Ge J. PDGFR-β-activated ACK1-AKT signaling promotes glioma tumorigenesis. Int J Cancer 2015; 136: 1769-1780.
32. Zhang Sh, Lai N, Liao K, Sun J, Lin Y. MicroRNA-210 regulates cells proliferation and apoptosis by targeting regulator of differentiation 1 in glioblastoma cells. Folia Neuropathol 2015; 3: 236-244.

Original paper

Folia Neuropathologica

Effect of recombinant *Lactococcus lactis* producing myelin peptides on neuroimmunological changes in rats with experimental allergic encephalomyelitis

Kaja Kasarełło[1,2*], Agnieszka Szczepankowska[3*], Barbara Kwiatkowska-Patzer[2], Andrzej W. Lipkowski[2], Roman Gadamski[4], Dorota Sulejczak[5], Magdalena Łachwa[6], Michał Biały[1], Jacek Bardowski[3]

**These authors contributed equally to this study*

[1]Department of Experimental and Clinical Physiology, Laboratory of Centre for Preclinical Research, Medical University of Warsaw, [2]Department of Neuropeptides, Mossakowski Reaserch Center, Polish Academy of Sciences, Warsaw, [3]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, [4]Department of Clinical and Experimental Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, [5]Department of Experimental Pharmacology, Mossakowski Medical Research Centre, Polish Academy of Sciences, [6]Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre, Polish Academy of Sciences, Poland

Folia Neuropathol 2016; 54 (3): 249-258 DOI: 10.5114/fn.2016.62534

Abstract

Multiple sclerosis (MS) is a human autoimmune neurodegenerative disease with an unknown etiology. Despite various therapies, there is no effective cure for MS. Since the mechanism of the disease is based on autoreactive T-cell responses directed against myelin antigens, oral tolerance is a promising approach for the MS treatment. Here, the experiments were performed to assess the impact of oral administration of recombinant Lactococcus lactis producing encephalogenic fragments of three myelin proteins: myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein, on neuroimmunological changes in rats with experimental allergic encephalomyelitis (EAE) – an animal model of MS.

Lactococcus lactis whole-cell lysates were administered intragastrically at two doses (10^3 and 10^6 colony forming units) in a twenty-fold feeding regimen to Lewis rats with EAE. Spinal cord slices were subjected to histopathological analysis and morphometric evaluation, and serum levels of cytokines (IL-1b, IL-10, TNF-α and IFN-γ) were measured. Results showed that administration of the L. lactis preparations at the tested doses to rats with EAE, diminished the histopathological changes observed in EAE rats and reduced the levels of serum IL-1b, IL-10 and TNF-α, previously increased by evoking EAE. This suggests that oral delivery of L. lactis producing myelin peptide fragments could be an alternative strategy to induce oral tolerance for the treatment of MS.

Key words: *MS, autoimmunity, oral tolerance, Lactococcus lactis, EAE, myelin proteins.*

Communicating author

Kaja Kasarełło, Department of Experimental and Clinical Physiology, Laboratory of Centre for Preclinical Research, Medical University of Warsaw, 1b Banacha St, 02-097 Warsaw, Poland, phone: +48 22 116 6113, e-mail: kaja.kasarello@wum.edu.pl

Introduction

Multiple sclerosis (MS) is an autoimmune disease that targets young people (20-40 years old) and is more common in women than in men. It is most frequently encountered in Northern Europe and North America, affecting approximately 1 in 1000 people. The genesis of MS remains unknown, but some evidence has supported the contributions of genetic and environmental factors or viral infection [35]. Despite its unidentified origin, the mechanism of MS involves humoral and cellular immunity and is based on T-cell autoreactivity directed against myelin antigens. Autoreactive T-lymphocytes migrate through the open blood-brain barrier into the central nervous system (CNS), where they recognize myelin proteins as pathogenic and induce an antigen-specific inflammatory process. Inflammation directed against myelin proteins causes demyelination and further deterioration of axons or even whole neurons, which results in deceleration or disruption of neurotransmission [12]. Tissue injury also affects oligodendrocytes function due to glutamatergic excitotoxicity in the sites of inflammatory lesions [10]. CNS damage provokes progressive physical disability and associated mental impairment that occur in MS patients. Currently, there is no effective therapy for MS. The most common treatment is systemic immunosuppression, which may also weaken the immune system and expose the patient to frequent infections [3,12,42]. However, new therapies targeting specific mechanisms involved in the pathology of MS, such as natalizumab, alemtuzumab or daclizumab may cause severe side effects or result in lower efficiencies than previously expected [7-9]. Thus, there is a need to identify novel therapies that are more effective and safe for patients. Among the most promising approaches developed in recent years that could potentially be applied for the treatment of autoimmune diseases is the induction of oral tolerance [11].

Oral tolerance is a natural state of a decreased immunoresponse to the previously fed antigen. The mechanism induces tolerance toward ingested antigens, which are normally encountered in the body (e.g. commensal bacteria, food). The phenomenon of oral tolerance has been applied for ovalbumin-induced anaphylaxis, contact hypersensitivity or the suppression of antibody production. The same approach has been also proposed for the treatment of autoimmune diseases, and many preclinical trials, including MS therapy, have been conducted [11,37,38]. In most of those trials, myelin basic protein (MPB) or its fragments served as the antigen, and all were shown to reduce the clinical symptoms of induced experimental allergic encephalomyelitis (EAE) [15,26]. However, MBP is not the only antigen that is considered to be immunogenic in MS. Antibodies directed against proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been detected in the cerebrospinal fluid of patients with MS [1,17]. Previously, in our laboratory, experiments with the use of pig spinal cord hydrolysate as the source of broad spectrum of myelin antigens to evoke oral tolerance in rats were performed [18]. In the presented experiment, we decided to use the most encephalogenic fragments of these three proteins, MBP85-97, PLP139-151 and MOG35-55, to induce a state of oral tolerance [2,23].

Since peptide synthesis is expensive and involves many complicated chemical procedures, other techniques for obtaining peptides are desirable. One example is the application of food-grade lactic acid bacteria (LAB) as biofactories for the production of heterologous proteins [28]. Lactic acid bacteria are present in fermented foods, are safe for human and animal consumption, and are extensively developed as live antigen delivery systems. Heterologous expression of therapeutic proteins, such as enzymes, cytokines or antigens, allows the broad use of these bacteria in various applications [5]. For our purposes, we designed recombinant *Lactococcus lactis* strains for use as a mucosal antigen delivery system. As LAB are present naturally in the gastrointestinal tract, we aimed to develop a minimally invasive method to induce oral tolerance that could be further applied for MS treatment.

In our experiments, EAE was applied as an animal model of MS. As EAE is a predominantly used MS model that is based on autoimmunological aspects of the disease, it was appropriate for our investigations. Also Lewis rats used in this study are commonly employed in such experiments, being one of the strains susceptible for EAE [23,39].

Our previous results demonstrated that feeding animals a mixture of *L. lactis* lysates producing MBP85-97, PLP139-151 and MOG35-55, in a twenty-fold but not a four-fold feeding scheme, could diminish the clinical symptoms of EAE [19]. In this study, we examined the influence of feeding *L. lactis* lysates on histo-

Table I. Strains and plasmids

Strain	Genotype	Source
Lactococcus lactis		
IBB360	Natural isolate, characterized as an autolytic strain	IBB PAS collection
Plasmids		
pIL253:PptcB	Ery^R, pIL253 derivative with regulated *ptcB* gene promoter region	[19]
pIL253:PptcB:MOG35-55	Ery^R, pIL253 derivative with *ptcB* gene promoter, expressing MOG35-55 antigen	[19]
pIL253:PptcB:MBP85-97	Ery^R, pIL253 derivative with *ptcB* gene promoter, expressing MBP85-97 antigen	[19]
pIL253:PptcB:PLP139-151	Ery^R, pIL253 derivative with *ptcB* gene promoter, expressing PLP139-151 antigen	[19]

pathological changes in the CNS and serum cytokine levels in rats with EAE.

Material and methods

Animals

Three-month-old female Lewis rats, provided by the Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, weighing approximately 180 g were used in the study. The animals were provided with food and water ad libitum unless otherwise required. Experiments were conducted in accordance with EU Directive 2010/63/EU for animal experiments and based on the consent of the Fourth Local Ethics Committee for Animal Experiments in Warsaw (Act no. 66/2010 of 08.10.2010).

Bacterial strains and plasmids

Recombinant *L. lactis* derivatives of the natural isolate IBB360, and plasmids that were used in this work are listed in Table I. Construction of the pIL253:PptcB recombinant vectors expressing myelin antigens MBP85-97, PLP139-151 and MOG35-55 have been described elsewhere [19].

Preparation of whole-cell lysates of recombinant lactococci

Recombinant lactococci were prepared for intragastric administration as follows. Cells were grown overnight at 30°C in M17 medium [36] supplemented with 0.5% cellobiose and erythromycin (5 µg/ml). Subsequently, cells were harvested (8000 g, 10 min., 4°C) and washed once with 0.9% NaCl. Cells were then suspended in 0.9% NaCl and disrupted 3 times for 1 min using the MiniBeadbeater (BioSpec Products) and 106-µm glass beads (Sigma). Adequate dilutions of cell lysates were generated that corresponded to doses of 10^3 and 10^6 colony forming units (CFUs). Single doses were frozen in Eppendorf tubes in liquid nitrogen and stored at –20°C until use.

Study design

A total number of 36 animals were randomly divided into 6 groups, consisting of 6 rats each. Animals with evoked EAE were fed intragastrically. Animals were given preparations containing either bacteria with empty vector (pIL253:PptcB) or a mixture of bacteria producing myelin peptides (MOG35-55, MBP85-97 and PLP139-151). Both preparations were administered in two doses. As reference, a group of animals with EAE, not receiving the bacteria preparations was created. Non-treated animals served as a control group. For the time diagram of the experiment see Figure 1.

Animal feeding

Using a gauged pointed needle, animals were fed intragastrically with bacteria preparations once daily for 20 consecutive days. Animals were deprived of food two hours before administration of the bacteria. The administered preparations contained whole-cell *L. lactis* lysates diluted accordingly in 0.5 ml saline to obtain a dose of 10^3 or 10^6 CFU/rat.

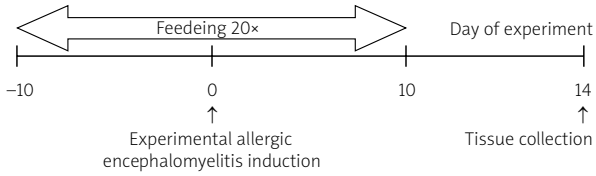


Fig. 1. Time diagram of experiment.

Experimental allergic encephalomyelitis

Animals were immunized on the 10th day of feeding. The day of immunization was defined as 0 days post-immunization (DPI). EAE was evoked as follows: slightly anesthetized animals (intraperitoneal injection with sodium brietal, Lilly) were injected intradermally into the hind paws with 100 µl (per paw) of the immunization mixture. The mixture contained guinea pig spinal cord homogenate (50% homogenate in PBS) with Freund's adjuvant (1 : 1) and *Mycobacterium tuberculosis* (4 mg/ml). The components were mixed well before application.

Material collection

At 15 DPI, the animals were anesthetized (0.67 ml/kg ketamine + 0.5 ml/kg xylazine *i.p.*, Vetoquinol Biowet) and, after blood collection, perfused transcardially with 4% paraformaldehyde. Lumbar and cervical segments of spinal cords were collected from the spinal canal for histopathological analysis. Serum was obtained from the collected blood.

Histopathological analysis

The collected spinal cord fragments were embedded in paraffin and cut into 8-µm-thick slices. Cresyl violet staining (1% water solution) was performed for cell exposure. A series of labeling experiments were also performed using the sections with the following antibodies: anti-T cells (Anti-CD45R0, DAKO), anti-GFAP (anti-glial fibrillary acidic protein, DAKO) and anti-endogenous albumin (rabbit IgG fraction against rat albumin, MP Biomedicals). The procedure was performed as follows: using dewaxed slices, endogenous peroxidase was blocked with 3% hydrogen peroxide, and the background was blocked with 10% albumin. After incubation with the primary antibodies, the sections were washed and then incubated with a secondary antibody coupled to biotin. After washing, streptavidin coupled to peroxidase was applied followed by a chromogen. The sections were then stained with Mayer's hematoxylin. The chemicals were supplied by DAKO. The sections were analyzed using a light microscope (AH3, Olympus) and images were obtained using a designated camera (3-35AD-4, Olympus).

Morphometric analysis

The morphometric analysis was conducted using both lumbar and cervical spinal cord segment sections stained with Cresyl violet. The area of the whole spinal cord cross-section and the area occupied by the inflammatory infiltrate were measured to determine the ratio, which is presented as the percentage of the slice area occupied by the inflammatory infiltrate.

Measurement of cytokine levels

In serum obtained from collected blood, cytokine levels (IL-1b, IL-10, TNF-α and IFN-γ) were analyzed. Analysis were made using the Bio-Plex Rat Cytokine Assay (Bio-Rad, Poland) according to the protocol supplied by the manufacturer (Bio-PlexTM Cytokine Assay Instruction Manual, Bio-Rad, Poland). Measurements were made using Luminex Bio Plex 200 analyzer, equipped with the software Manager version 5.0. (Bio-Rad, Poland).

Statistical analysis

The results are presented as means ± SD. Statistical assessments were performed using the Mann-Whitney test. $P < 0.05$ was considered statistically significant.

Results

Histopathological studies

Spinal cord cross-sections from control animals showed no signs of inflammation (Fig. 2A and D). Inflammatory infiltrates that were visible in both the gray and white matter of the spinal cord in rats with evoked EAE (Fig. 2B) diminished after oral administration of both doses of *L. lactis* lysates (Fig. 2C). Moreover, there were no visible differences between the intensity of the infiltrates in rats fed preparations containing lysates of bacteria producing myelin peptides and control preparations (data not shown). We also noticed that the infiltrates in animals fed bacterial lysates (both the control and the *L. lactis* producing myelin peptides preparations) had a reduced abundance of inflammatory cells (Fig. 2F) compared with rats with EAE (Fig. 2E), yet they occupied a similar area.

Anti-GFAP labeling revealed active astrocytes in both the gray and white matter of the spinal cord. In untreated rats, a weak reaction was observed, and the astrocytes displayed short tabs and insufficient cytoplasm (Fig. 2G), whereas rats with induced EAE were characterized by active astrocytes with long, branched tabs (Fig. 2H). After oral administration

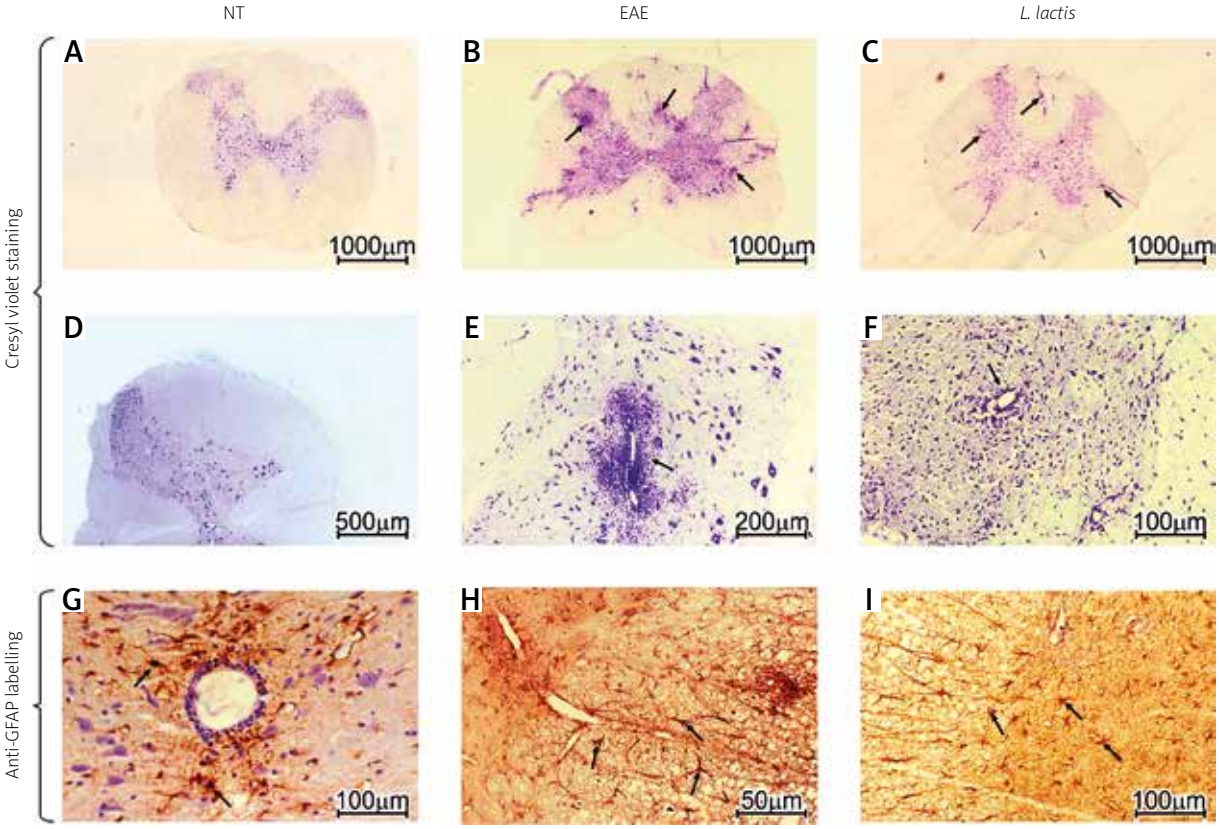


Fig. 2. Spinal cord cross-sections. Cresyl violet staining (**A–F**); inflammatory infiltrates are indicated by arrows. Anti-GFAP labeling (**G–I**); astrocytes are indicated by arrows. Non-treated animals (NT), animals with induced experimental allergic encephalomyelitis (EAE), animals with EAE fed bacteria producing myelin peptides (*Lactococcus lactis*), *n* = 6.

of *L. lactis* lysates, the number of active astrocytes increased in rats with EAE (Fig. 2I).

Anti-endogenous albumin labeling revealed albumin in the perivascular area of rats with induced EAE (Fig. 3A). T cells were observed in the area occupied by inflammatory infiltrates (Fig. 3B).

Morphometric analysis

A morphometric analysis was conducted to assess the area of the spinal cord cross-section occupied by inflammatory infiltrates. No significant differences were observed between groups of fed or non-fed rats with EAE. Animals in the experimental

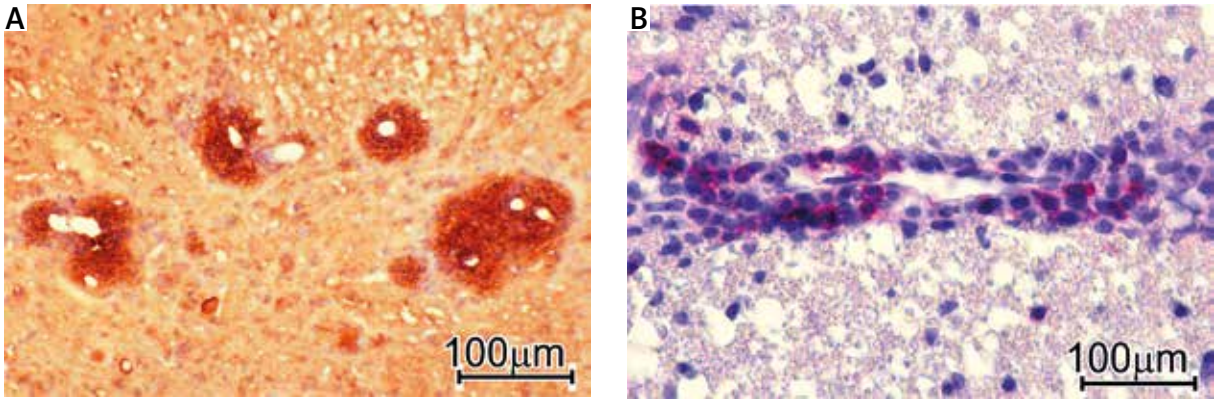


Fig. 3. Spinal cord cross-sections. Anti-endogenous albumin labeling (**A**). Anti-T cell labeling (**B**), *n* = 6.

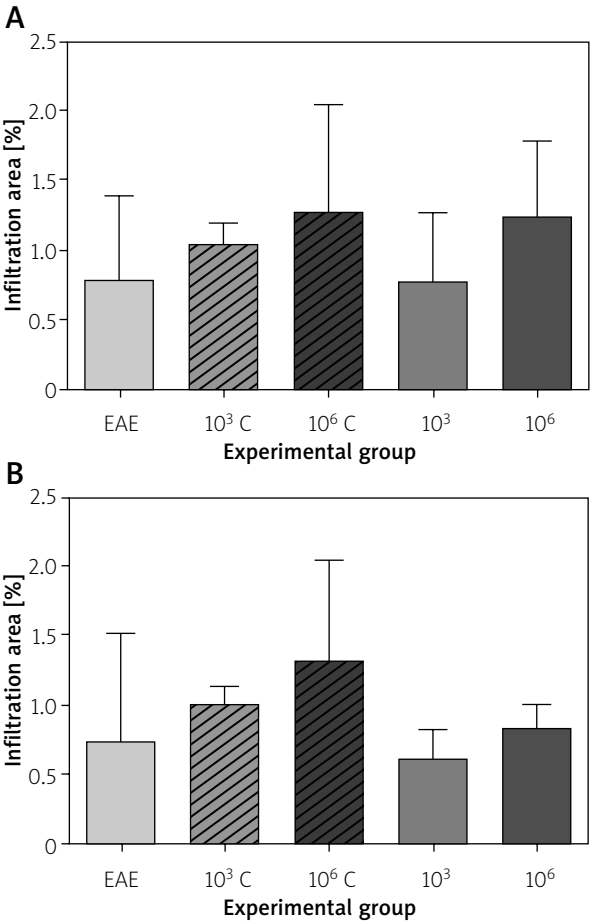


Fig. 4. Percentage of the section area occupied by inflammatory infiltrates. Cervical segment of spinal cord (**A**). Lumbar segment of spinal cord (**B**), $n = 6$.

groups presented very large individual differences in the infiltration area, resulting in highly dispersive results. Therefore, the changes observed in the area occupied by inflammatory infiltrates can only be described as trends and were similar for both cervical and lumbar spinal cord segments (Fig. 4A and B). In rats that received a lower dose of the lysate (10^3) of myelin peptide-producing bacteria, inflammatory infiltrates occupied a small area of the spinal cord cross-section, while higher dose preparations of both control (10^6 C) and myelin peptide-producing bacteria (10^6) lead to a slight increasing trend in the area occupied by inflammatory infiltrates in comparison to non-fed rats with EAE.

Cytokine levels

The induction of EAE in rats resulted in elevated levels of IL-1b, IL-10, TNF-α and IFN-γ compared with non-treated animals (Fig. 5A-D). Oral administration of *L. lactis* preparations (control and bacteria producing myelin peptides) at both doses decreased IL-1b, IL-10 and TNF-α to levels that were similar to non-treated animals (Fig. 5A-C). The same effect was observed for rats that received control bacterial preparation. However, in the group that was fed lysates from myelin-producing bacteria, the level of IFN-γ remained elevated and even increased slightly following the administration of preparation at the higher dose (Fig. 5D).

Discussion

Multiple sclerosis is a disease of unknown etiology. As a result, only the symptoms, and not the cause, can be treated. Current MS therapies include mostly non-specific, systemic immunosuppression, which is not free from side effects or can simply be inefficient. Other therapies may be more adequate, but they may also cause severe side effects (e.g. natalizumab) [42]. Our intention in the present study was to exploit the naturally occurring state of oral tolerance for the treatment of MS. As MS is an autoimmune disease in which autoreactive T lymphocytes destroy the myelin sheath, the induction of oral tolerance, which decreases the systemic immunoresponse to previously fed antigen, may be an interesting therapeutic alternative. Gut-associated lymphoid tissue (GALT) produce anti-inflammatory cytokines providing the immunosuppressive environment, helping to maintain the state of tolerance for antigens from food or commensal bacteria. Dendritic cells in the GALT present the gut-derived antigens to naïve T cells in Peyer's patches, what results in differentiation of T cells into regulatory T cells (Tregs) producing IL-10 and TGF-β, or internalization of TCR leading to anergy. Thus, orally administered fragments of myelin peptides induce, depending on the antigen dose, active suppression (inhibiting of Th1-dependent immunoresponse) or anergy/clonal deletion of responsive T lymphocytes (both Th1 and Th2) in the mucosal immune system [4,27,40]. Anergic T cells are not able to migrate from the site of tolerance induction, and remaining in the GALT produce anti-inflammatory cytokines. Tregs induced in the Peyer's patches pass into the bloodstream and spread the antigen-specific tolerance throughout the body. Additionally, apoptosis of autoreactive T cells is induced in the thymus after oral administration of the autoantigen [27,34]. This process results in

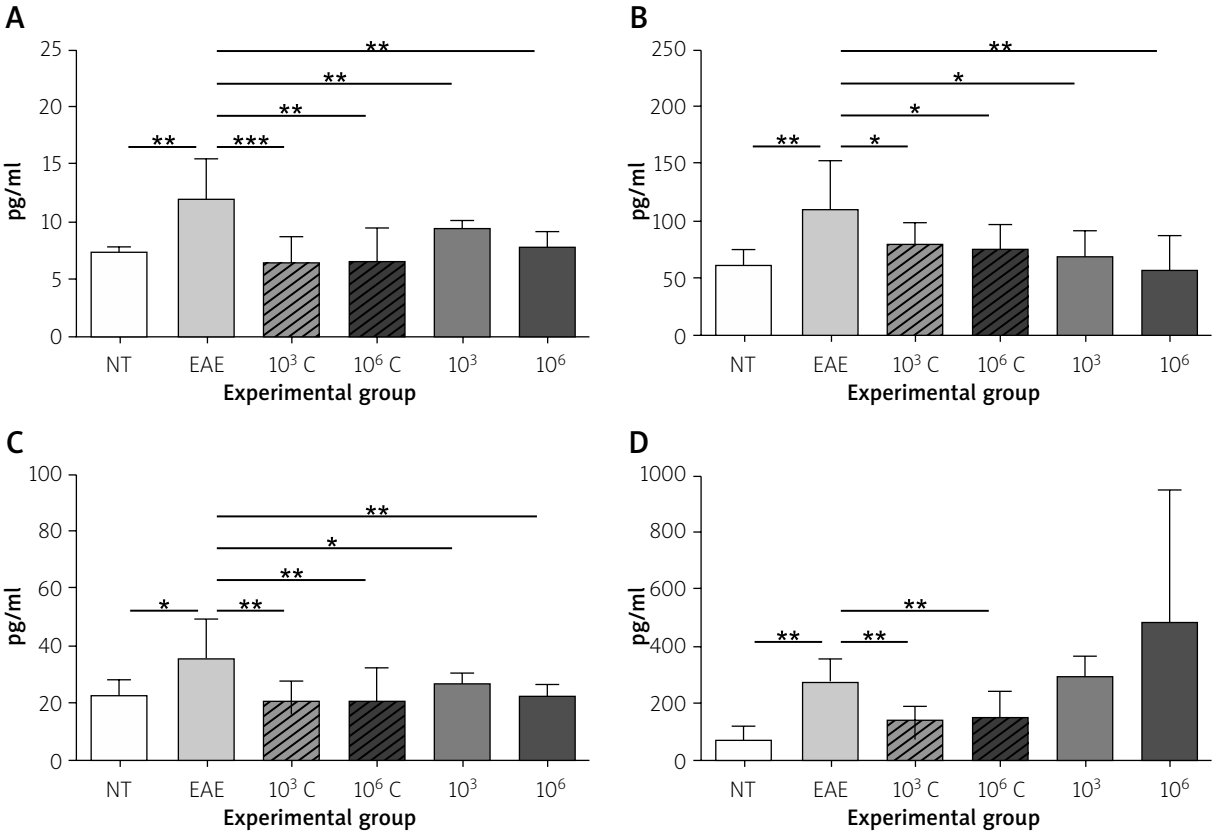


Fig. 5. Serum cytokine levels. Levels of IL-1b (**A**), IL-10 (**B**), TNF-α (**C**), and IFN-γ (**D**), *n* = 6.

a diminished immunoresponse against myelin peptides. Such an approach is anticipated to lead to an antigen-specific, non-systemic, non-specific immunosuppression for the treatment of MS. In addition to oral tolerance, mucosal-associated tolerance (e.g. respiratory tract), generally known as mucosal tolerance, has been considered for many decades as a potential approach for the treatment of illnesses such as uveitis, diabetes, rheumatoid arthritis or allergy [38,41]. Immunological tolerance is an important mechanism that protects the organism from developing an immunoresponse to the exogenous antigens that constantly contact the mucosa, by treating them as self-antigens. Oral tolerance in particular is responsible for maintaining tolerance to dietary antigens and gut microflora. *Lactococcus lactis* bacteria are non-invasive, non-pathogenic and non-colonizing microorganisms with a GRAS (Generally Regarded As Safe) status. There is a large amount of evidence supporting the utility of these bacteria as heterologous protein expression systems. Recombined, leptin-secreting *L. lactis* administered intranasally in mice resulted in a decrease in body mass and food intake [6]. Additionally, feeding with ovalbumin-secreting *L. lactis* has been reported to induce ovalbumin tolerance in mice. Furthermore, mice that received *L. lactis* alone were able to mount a slight immune response, indicating that this bacterial species itself can enhance the induction of oral tolerance [16,25].

We have previously shown that twenty-fold, but not four-fold, oral administration of a mixture of lysates from *L. lactis* producing MBP85-97, PLP139-151 and MOG35-55 in two doses of 10^3 and 10^6 CFU per dose reduces clinical symptoms and body mass drop in rats with EAE [19]. Here, we describe the effects of bacterial lysates on some neuroimmunological parameters of induced EAE, an animal model of MS. During the progression of EAE, the blood-brain barrier opens. This phenomenon is consistent with the theory regarding the mechanism responsible for the development of MS, in which autoreactive T cells migrate into the CNS and induce an inflammatory response against myelin epitopes [13,24]. Our histopathological analysis of the spinal cord cross-sections demonstrated that evoking EAE resulted in the appearance of perivascular infiltrates in the CNS. Anti-endoge-

nous albumin labeling revealed its proximity to the vessels, confirming the opening of the blood-brain barrier. T-cell labeling showed that the infiltrates consisted of those immune cells. In our experiments, the inflammatory process observed after evoking EAE in rats was diminished by oral administration of *L. lactis* lysates. The decrease in inflammation intensity was manifested by a reduced number of immune cells rather than by a smaller infiltration surface.

Anti-GFAP labeling was performed to expose active astrocytes. The induction of EAE resulted in an increase in the activity of a greater number of astrocytes, as compared with control rats, and feeding the animals with bacterial lysates further increased the number of these cells. This phenomenon could account for the increased astrocyte proliferation associated with repair processes and glial scar formation in the CNS [26,30].

Serum cytokine levels were measured to determine the peripheral immunological state of the animals [20,31,33]. The results showed that the induction of EAE resulted in an increase in all measured cytokine levels, including both pro- and anti-inflammatory cytokines. Feeding animals with recombinant *L. lactis* lysates decreased IL-1b, IL-10 and TNF-α to the levels detected in non-treated animals, which could imply that animals fed bacterial preparations did not develop an immune reaction. However, this explanation is inconsistent with the histopathological analysis showing infiltration into the CNS. CNS inflammation must be a result of a peripheral process because the immunization mixture was injected peripherally (paw pads). Therefore, it is more likely that our results capture the moment when the inflammatory process was completely cleared in the periphery but still present in the CNS. Differences in the reaction time of the peripheral versus the central immune system may result from the initial induction of antigen tolerance in the gut mucosa, followed by the periphery and then the CNS [29].

Interesting effects have been documented with respect to IFN-γ. After EAE induction, IFN-γ was elevated in comparison to non-treated animals, and feeding with control bacterial preparations resulted in decreased IFN-γ levels. However, when the animals were fed either dose of the bacterial lysate producing myelin peptides, the level of IFN-γ remained elevated. Previous investigations have shown that IFN-γ influences apoptosis of autoreactive T cells by astrocytes [14,32], which may underlie the reduced number of inflammatory cells observed in the infiltrates in rats fed bacterial lysates.

An important observation was made with respect to the effect of oral administration of lysates containing *L. lactis* cells carrying the empty vector (pIL253:PptcB). Results showed that the control preparation may produce a similar but less intense effect in rats as lysates from bacteria producing myelin antigens. It is known that lactococcal cells themselves are able to enhance immunotolerance in mucosa. Our observations are similar to those of Lavasani *et al*. [22] who showed that, due to their immunomodulatory activity and ability to induce regulatory T cells, three probiotic lactic acid bacteria strains (*L. paracasei*, *L. plantarum* DSM 15312 and *L. plantarum* DSM 15313) could be helpful for the treatment of autoimmune diseases.

Difficulties associated with the development of effective MS therapy are linked to the observation that almost every patient presents a unique course of the disease [21]. Similarly, in experimental animals, even inbred animal lines, the clinical symptoms will differ among individual cases. In our trials, EAE was induced using guinea pig spinal cord homogenate as the source of myelin antigens to imitate MS, in which many encephalogenic antigens are believed to play a role. Our previous study has also shown that the three main myelin peptide fragments selected to induce oral tolerance appear to be effective [19].

Experimental results in this work indicate that *L. lactis* is a good antigen delivery system for the induction of oral tolerance in animals with EAE. This notion is confirmed not only by the diminished clinical symptoms of the ongoing disease but also by the decreased inflammatory activity. We suggest that oral administration of lysates from recombinant *L. lactis* cells accelerates the resolution of peripheral inflammation, decreases the intensity of inflammation in the CNS, and boosts the repair process. Considered together, these phenomena may elucidate a perspective for further development of this approach in MS therapy.

Acknowledgements

This work was supported by the Ministry of Science and Higher Education grant no. N302 009 32/1139.

First author has been supported by a scholarship from the European Social Fund, Human Capital Operational Programme.

Disclosure

Authors report no conflict of interest.

References

1. Abott JD, Blanchfield JL, Martinson DA, Russel SC, Taslim N, Curtis AD, Mannie MD. Neuroantigen-specific, tolerogenic vaccines: GM-CSF is a fusion partner that facilitates tolerance rather than immunity to domminant self-epitopes of myelin in murine models of experimental autoimmune encephalomyelitis (EAE). BMC Immunol 2011; 12: 72.
2. Adelmann M, Wood J, Benzel I, Fiori P, Lassman H, Matthieu JM, Gardinier MV, Dornmair K, Linington C. The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) induces acute demyalinating experimental autoimmune encephalomyelitis in the Lewis rats. J Neuroimmunol 1995; 63: 17-27.
3. Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, Han M, Zhong F, Castellanos JG, Mair R, Christakos A, Kolkowitz I, Katz L, Killestein J, Polman CH, de Waal Malefyt R, Steinman L, Raman C. T helper type 1 and 17 cells determine efficacy of IFN-β in multiple sclerosis and experimental autoimmune encephalomyelitis. Nat Med 2010; 16: 406-412.
4. Benson JM, Campbell KA, Guan Z, Gienapp IE, Stuskman SS, Forsthuber T, Whitacre CC. T-cell activation and receptor downmodulation precede deletion induced by mucosally administered antigen. J Clin Invest 2000; 106: 1031-1038.
5. Bermudez-Humaran LG. Lactococcus lactis as a live vector for mucosal delivery of therapeutic proteins. Hum Vaccin 2009; 5: 264-267.
6. Bermudez-Humaran LG, Nouaille S, Zilberfarb V, Corthier G, Cruss A, Langella P, Issad T. Effects of intranasal administration of a leptin-secreting Lactococcus lactis recombinant on food intake, body weight, and immune response of mice. Appl Environ Microbiol 2007; 73: 5300-5307.
7. Bielekova B, Richert N, Blevins G, Markovic-Plese S, McCartin J, Frank JA, Würfel J, Ohayon J, Waldmann TA, McFarland HF, Martin R. Humanized anti-CD25 (daclizumab) inhibits disease activity in multiple sclerosis patients failing to respond to interferon β. Proc Natl Acad Sci U S A 2004; 101: 8705-8708.
8. Castro-Borrero W, Graves D, Frohman TC, Bates Flores A, Hardeman P, Logan D, Orchard M, Greenberg B, Frohman EM. Current and emerging therapies in multiple sclerosis: a systemic review. Ther Adv Neurol Disord 2012; 5: 205-220.
9. Coles AJ. Alemtezumab therapy for multiple sclerosis. Neurotherapeutics 2013; 1: 29-33.
10. Dąbrowska-Bouta B, Stróżyńska L, Chalimoniuk M, Frontczak-Baniewicz M, Sulkowski G. The influence of glutamatergic receptor antagonists on biochemical and ultrastructural changes in myelin membranes of rats subjected to experimental autoimmune encephalomyelitis. Folia Neuropathol 2015; 53: 317-326.
11. Faria AMC, Weiner HL. Oral tolerance: implications for autoimmune diseases. Clin Dev Immunol 2006; 13: 143-157.
12. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KHG. T cells in multiple sclerosis and experimental allergic encephalomyelitis. Clin Exp Immunol 2010; 162: 1-11.
13. Franklin RJ, Ffrench-Constant C, Edgar JM, Smith KJ. Neuroprotection and repair in multiple sclerosis. Nature reviews. Neurology 2012; 8: 624-634.
14. Hara H, Nanri Y, Tabata E, Mitsutake S, Tabira T. Identification of astrocyte-derived immune suppressor factor that induces apoptosis of autoreactive T cells. J Neuroimmunol 2011; 233: 135-146.
15. Higgins PJ, Weiner HL. Supression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. J Immunol 1988; 140: 440-445.
16. Huibregste IL, Snoeck V, de Creus A, Braat H, De Jong EC, Van Deventer SJ, Rottiers P. Induction of ovalbumin-specific tolerance by oral administration of Lactococcus lactis secreting ovalbumin. Gastroenterology 2007; 133: 517-528.
17. Jurynczyk M, Walczak A, Jurewicz A, Jesionek-Kupnicka D, Szczepanik M, Selmaj K. Immune regulation of multiple sclerosis by transdermally applied myelin peptides. Ann Neurol 2010; 68: 593-601.
18. Kasarello K, Gadamski R, Piotrowski P, Kurzepa K, Kwiatkowska-Patzer B, Lipkowski AW. Effect of oral administration of pig spinal cord hydrolysate on clinical and histopathological symptoms of experimental allergic encephalomyelitis in rats. Folia Neuropathol 2015; 53: 128-138.
19. Kasarello K, Kwiatkowska-Patzer B, Lipkowski AW, Bardowski JK, Szczepankowska AK. Oral administration of Lactococcus lactis expressing synthetic genes of myelin antigens in decreasing experimental autoimmune encephalomyelitis in rats. Med Sci Monit 2015; 21: 1587-1597.
20. Lalor SJ, Dungan LS, Sutton CE, Basedo SA, Fletcher JM, Mills KH. Caspase-1-processed cytokines IL-1β and IL-18 promote IL-17 production by γδ and CD4 T cells that mediate autoimmmunity. J Immunol 2013; 186: 5738-5748.
21. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. Nature Reviews. Neurology 2012; 8: 647-656.
22. Lavasani S, Dzhambazov B, Nouri MA, Fak F, Buske S, Molin G, Thorlacius H, Alenfall J, Jeppsson B, Weström B. A novel probiotic mixture exerts a therapeutic effect on expreimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. PLoS One 2010; 5: 1-11.
23. Link H, Xiao BG. Rat models as tool to develop new immunotherapies. Immunol Rev 2001; 184: 117-128.
24. Lyck R, Engelhardt B. Going Against the tide – how encephalogenic T cells breach the blood-brain barrier. J Vasc Res 2001; 49: 4497-4509.
25. Maillard MH, Snapper SB. Teaching tolerance with a probiotic antigen delivery system. Gastroenterology 2007; 133: 706-709.
26. Meyer AL, Benson JM, Giennap IE, Cox KL, Whitacre CC. Supression of murine chronic relapsing experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. J Immunol 1996; 157: 4230-4238.
27. Mirenda V, Millington O, Lechler RI, Scott D, Hernandez-Fuentes MP, Read J, Tan PH, George AJT, Garside P, Marelli-Gerg FM. Tolerant T cells display impaired trafficking ability. Eur J Immunol 2005; 35: 2146-2156.
28. Morello E, Bermudez-Humaran LG, Llull D, Sole V, Miraglio N, Langella P, Poquet I. Lactococcus lactis, an efficient cell fac-

tory for recombinant protein production and secretion. J Mol Microbol Biotechnol 2008; 14: 48-58.
29. Pabst O, Mowat AM. Oral tolerance to food protein. Mucosal Immunol 2012; 5: 232-239.
30. Reynolds R, Roncaroli F, Nicholas R, Radotra B, Gveric D, Howell O. The neuropathological basis of clinical progression in multiple sclerosis. Acta Neuropathol 2011; 122: 155-170.
31. Rodgers JM, Miller SD. Cytokine control of inflammation and repair in the pathology of multiple sclerosis. Yale J Biol Med 2012; 85: 447-468.
32. Sanvito L, Constantinescu CS, Gran B, 't Hart BA. The Multifaceted Role of Interferon-γ in Central Nervous System Autoimmune Demyelination. The Open Autoimmunity Journal 2010; 2: 151-159.
33. Sharief MK, Hentges R. Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. N Engl J Med 1991; 325: 467-472.
34. Song F, Wardrop RM, Giennap IE, Stuckman SS, Meyer AL, Shawler T, Whitacre CC. The Peyer's patch is a critical immunoregulatory site for mucosal tolerance in experimental autoimmune encephalomyelitis (EAE). J Autoimmun 2008; 30: 230-237.
35. Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol 2005; 23: 683-747.
36. Terzaghi BE, Sandine WE. Improved medium for lactic streptococci and their bacteriophages. Appl Microbiol 1975; 29: 807-813.
37. Wardrop RM, Whitacre CC. Oral tolerance in the treatment of inflammatory autoimmune diseases. Inflamm Res 1999; 48: 106-119.
38. Weiner HL. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. Immunol Today 1997; 18: 335-343.
39. Wekerle H, Kurschus FC. Animal models for multiple sclerosis. Drug Discov Today: Disease Models 2006; 3: 359-367.
40. Wells JM, Rossi O, Meijerink M, Baarlen van P. Epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A 2011; 108: 4607-4614.
41. Whitacre CC, Gienapp IE, Orosz CG, Bitar DM. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. J Immunol 1991; 147: 2155-2163.
42. Ziemssen T, Ziemmssen F. The role of the humoral immune system in multiple sclerosis (MS) and its animal model experimental allergic encephalomyelitis (EAE). Autoimmun Rev 2010; 4: 460-467.

Original paper

Folia Neuropathologica

Administration of vitamin D_3 induces CNPase and myelin oligodendrocyte glycoprotein expression in the cerebral cortex of the murine model of cuprizone-induced demyelination

Farhad Mashayekhi[1], Zivar Salehi[2]

[1]Department of Cell and Molecular Biology, Faculty of Biological Sciences, Tonekabon Branch, Islamic Azad University, Tonekabon, [2]Department of Genetics, Faculty of Medicine, Tonekabon Branch, Islamic Azad University, Tonekabon, IR Iran

Folia Neuropathol 2016; 54 (3): 259-264 DOI: 10.5114/fn.2016.62535

Abstract

In the central nervous system (CNS) the main proteins of myelin are proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and CNPase. Myelin oligodendrocyte glycoprotein is a minor component of the myelin sheath, but is an important autoantigen linked to the pathogenesis of multiple sclerosis (MS). CNPase is expressed exclusively by oligodendrocytes in the CNS, and the appearance of CNPase seems to be one of the earliest events of oligodendrocyte differentiation and myelination. In this study the effects of vitamin D on total protein concentration, CNPase and MOG expression in the cerebral cortex of the murine model of cuprizone-induced demyelination was investigated. The mice were treated by cuprizone for five weeks in order to induce demyelination. The mice were then divided into 3 groups. The first group was injected intraperitoneally (IP) with vitamin D diluted in olive oil in the amount of 5 µg/kg/daily body weight. The second group (SHAM) was injected IP with olive oil and the third group was left without any injection as the control group (n = 11 for each group). After five weeks the mice were killed and the cerebral cortex was collected and the expression of CNPase and MOG was studied by Western blot. Total protein concentration in the vitamin D injected, SHAM and control groups were 0.918 ± 0.003, 0917 ± 0.004 and 0.916 ± 0.004 g/l, respectively (p > 0.05). However, a significant increase in the MOG and CNPase expression was seen in vitamin D injected group as compared to SHAM and control groups. It is concluded that vitamin D plays a role in the process of remyelination by increasing MOG and CNPase expression in the cortex.

Key words: *vitamin D, myelin, CNPase, MOG, cuprizone.*

Introduction

Multiple sclerosis (MS) is a neurodegenerative, inflammatory and demyelinating disease of the central nervous system (CNS) [11]. While the exact etiology of MS remains unknown, it is thought that many different genetic as well as environmental factors play a key role [18]. Hypovitaminosis D has long been considered as a risk factor for MS but there has recently been a sharp increase of interest in this factor [17,36]. The biologically active form of vitamin D is 1,25-dihydroxyvitamin D_3 that has a key role in the modulation of immune response [33]. This is suggested by the fact that many immune cells including

Communicating author

Prof. Farhad Mashayekhi, Department of Cell and Molecular Biology, Faculty of Biological Sciences, Tonekabon Branch, Islamic Azad University, Tonekabon, IR Iran, phone: 0098-9113330017, fax: 0098-131-3233647, e-mail: umistbiology@yahoo.co.uk

macrophages, activated B and T cells and dendritic cells contain vitamin D receptor (VDR). Vitamin D_3 is a potent immune modulator that can even cure the animal model with MS [5]. It has been shown that vitamin D_3 acts on myelination via the activation of several myelin-associated genes [4]. It was demonstrated that vitamin D_3 could actually promote the repair process in the cuprizone model of mice [26]. Vitamin D which is a peripheral regulator of Ca^{2+} homeostasis, has numerous other physiological functions including protection against certain immune mediated disorders including MS [8]. It was shown that vitamin D_3 may be able to suppress the inflammatory ways that lead to the progression of MS [31]. Elevated levels of vitamin D have been shown to be associated with an improvement in experimental autoimmune encephalomyelitis (EAE) [32].

In myelin, a number of structural classes of proteins are present. These include proteolipid protein (PLP), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), peripheral myelin protein 2 (P2), myelin-associated glycoprotein (MAG), myelin-associated oligodendrocytic basic protein (MOBP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) [9]. Myelin oligodendrocyte glycoprotein is a transmembrane protein present in the CNS myelin and it is also one of the main autoantigens in MS [20]. Mutations and/or polymorphisms in the MOG gene may contribute to the development and progression of MS [15]. Myelin oligodendrocyte glycoprotein is a potent encephalophatogen that triggers strong T-cell and B-cell responses [14]. CNPase was shown to be implicated as an autoantigen in MS and is expressed in oligodendrocytes and considered a marker for myelin forming cells [25,27].

Myelin oligodendrocyte glycoprotein is a transmembrane protein expressed on the surface of oligodendrocyte cells and on the outermost surface of myelin sheaths and speculated to serve as a necessary adhesion molecule to provide structural integrity to the myelin sheath and is known to develop late on the oligodendrocyte [2]. Myelin oligodendrocyte glycoprotein is a target antigen that leads to autoimmune-mediated demyelination. Myelin oligodendrocyte glycoprotein has received much of its laboratory attention in studies dealing with MS. Several studies have shown a role of antibodies against MOG in the pathogenesis of MS [10]. The aim of this study was to examine the *in vivo* effects of vitamin D_3 on the CNPase and MOG expression in the cerebral cortex of the murine model of cuprizone-induced demyelination.

Material and methods

Animals

Balb/c mice were purchased from the Pasteur Institute, Tehran, Iran and maintained on the light-dark (12 : 12) cycle beginning at 8.00 am. They were kept at a constant temperature in mice boxes with unrestricted access to laboratory food and water. The colony was maintained through random pair mating. Cage maintenance was performed once a week and the animals were handled by the same individuals throughout the experimental period. Food and tap water was available *ad libitum* throughout the acclimatization and experimental period. The work was undertaken according to the provisions of the Declaration of Helsinki (as revised in Brazil 2013). All animal protocols used have been approved by the authors' institutional animal experimentation committee. 33 female Balb/c mice aged 6 to 8 weeks were included in this study (n = 11 for each group).

Injection of demyelination and treatment with vitamin D_3

Demyelination was induced by feeding 8-10-week-old mice a diet containing 0.2% cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich Inc.) mixed into ground standard rodent chow. The cuprizone diet was administered for 5 weeks for demyelination. The control group received breeder chow without cuprizone admixture. Animals were then put on standard rodent chow without cuprizone to induce remyelination. Cuprizone, a copper chelator, induces demyelination in the corpus callosum, hippocampus, and some other white matter regions of the rodent CNS. Its underlying mechanism of demyelination is not well understood, but cuprizone has been used to induce CNS demyelination for many decades. It has been noted that mouse strain, age, or gender impact the degree of demyelination [22]. The mice were then divided into three groups. The first group was injected intraperitoneally (IP) with vitamin D_3 for 6 weeks in the amount of 5 µg/kg body weight diluted in olive oil by gavage daily. The second group (SHAM) was treated with the equivalent olive oil and the third group was left without any injection as the control group. After four weeks the cerebral cortex

was harvested after euthanasia by an intraperitoneal injection of an overdose of anesthetic (sodium pentobarbitone) and the cerebral cortex were removed and processed as described. In total 33 animals were used in this study (n = 11 for each group).

Cell extract

Fresh tissue samples (10 mg each) were chopped into tiny pieces and suspended in 0.5 ml of protein lysis buffer [150 mM NaCl, 1.0% NP40, 20 mM Tris (pH 7.5), 5 mM EDTA, and Complete Mini protease inhibitor cocktail (Roche Diagnostics Ltd., West Sussex, UK)] and then mechanically homogenized by sonication. After centrifugation, the protein extracts were recovered and stored at –70°C until they were used.

Total protein concentration and Western blotting

The total protein concentration in the cerebral cortex extracts was determined by the Bio-Rad protein assay based on the Bradford dye procedure. For Western blot, protein extracts (50 µg/lane) were separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Ltd. Hertfordshire, UK). The membranes were blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 5% dry milk and probed either with polyclonal anti-CNPase antibody (Abcam plc, Cambridge, UK); Anti-CNPase antibody (ab27695) (1 : 1000 dilution), monoclonal anti-MOG antibody (Abcam plc, Cambridge, UK; ab109746) (1 : 1000 dilution) or a mouse monoclonal anti-β-tubulin antibody (as a loading control) (Abcam plc, Cambridge, UK) (1 : 10 000 dilution) and then treated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein was visualized using the Enhanced Chemiluminescence western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was performed by scanning immunoblots and quantitating protein bands using an image analyzer (Metaview Software, V4.6.8, Fryer Company Inc.).

Statistical analysis

In order to assess a possible distortion in allele frequencies between cases and controls, we performed a χ^2 test with one degree of freedom for both allelic and genotypic distributions between the groups of cases and controls. Significant association was defined by $p \leq 0.05$.

Results

Total protein concentration

The total protein concentration in the cerebral cortex extracts from vitamin D_3 injected, SHAM and control groups was determined by the Bio-Rad protein assay based on the Bradford dye mixture. The total protein contents of vitamin D_3 injected, SHAM and control were 0.91 ± 0.004, 0.91 ± 0.004 and 0.91 ± 0.003 (g/l), respectively. No significant increase in the total protein concentration was seen in the vitamin D_3-injected brain samples compared with those from the SHAM and control groups ($p > 0.05$).

Analysis of CNPase and MOG expression by Western blotting

Western blot analysis was performed to quantitatively evaluate CNPase and MOG expression in the cerebral cortical extracts. A western blot analysis using anti-CNPase and -MOG antibodies as a probe confirmed the presence of CNPase and MOG in all the extracts. An image analyzer was used to determine the intensities of the band in the respective lanes. Quantification of the western blot bands from repeated experiments (n = 11) showed that the amount of CNPase and MOG was significantly increased in the vitamin D_3-injected cerebral cortical extracts when compared with SHAM and control groups ($p < 0.0001$). In the vitamin D-injected group CNPase expression was increased approximately 1.70 and 1.71 times versus control and SHAM groups, respectively. Meanwhile, MOG expression was increased approximately 1.31 and 1.43 times in the vitamin D-injected extracts versus control and SHAM groups, respectively (Figs. 1 and 2).

Discussion

Multiple sclerosis is characterized by focal myelin damage, oligodendrocyte loss and infiltration of macrophages and T lymphocytes [1]. While the etiology of MS remains unknown, it is thought that many different genetic as well as environmental factors play a role [18]. It has been demonstrated that there is a significant association between latitude, deliberate sun exposure and vitamin D supplementation

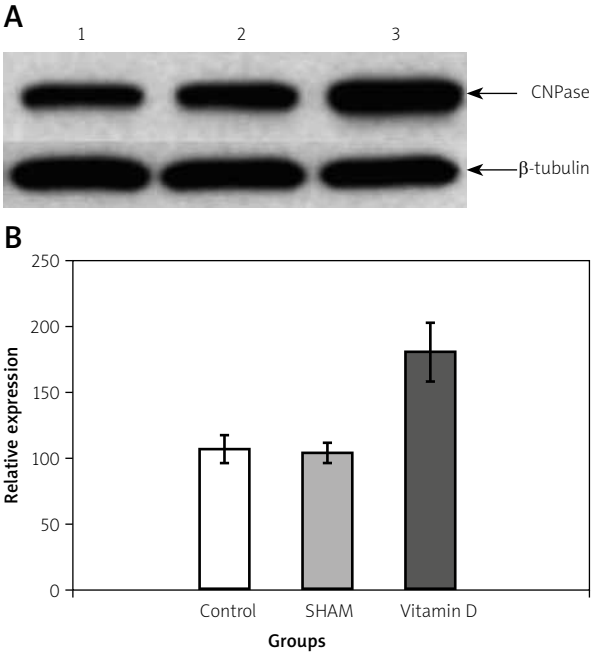


Fig. 1. A) CNPase expression in the cerebral cortex extracts from vitamin D_3 treated (Lane 3), SHAM (Lane 2) and control groups (Lane 1). β-tubulin (50-kDa) expression was determined as a protein loading control. **B**) Signal intensities from CNPase expression in the vitamin D_3 treated, SHAM and control cerebral cortex immunoblotting experiments were determined by densitometric analysis. In each of the experimental groups the number of animals investigated was n = 11. A significant increase in the CNPase expression was seen in vitamin D_3-injected group when compared with SHAM and control groups ($p < 0.0001$). No significant changes were seen between the SHAM and control group ($p = 0.91$).

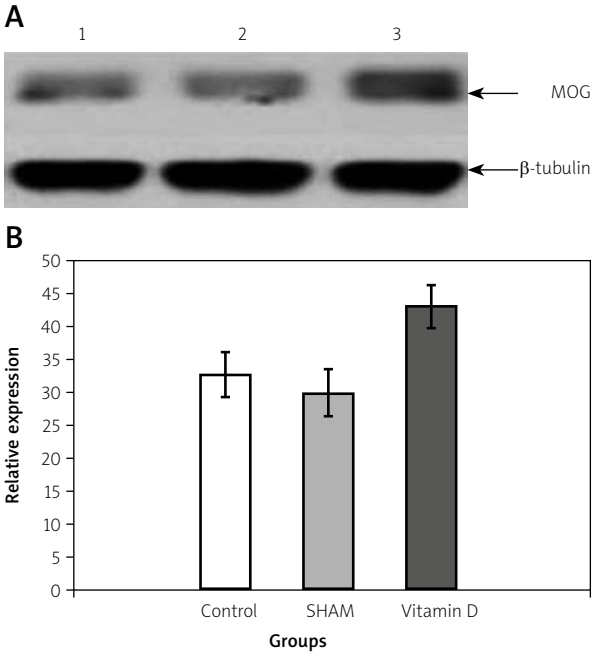


Fig. 2. A) MOG expression in the cerebral cortex extracts from vitamin D_3 treated (Lane 3), SHAM (Lane 2) and control groups (Lane 1). β-tubulin (50-kDa) expression was determined as a protein loading control. **B**) Signal intensities from MOG expression in the vitamin D_3 treated, SHAM and control cerebral cortex immunoblotting experiments were determined by densitometric analysis. In each of the experimental groups the number of animals investigated was n = 11. A significant increase in the MOG expression was seen in vitamin D_3-injected group when compared with SHAM and control groups ($p < 0.0001$). No significant changes were seen between the SHAM and control group ($p = 0.42$).

with MS [21]. In mice with EAE, only females could be treated with vitamin D_3 [32].

Nystad and colleagues showed that vitamin D could actually promote the repair process in a cuprizone-induced model of EAE mice, possibly by stimulating the effect on oligodendrocyte maturation and astrocyte activation [26]. It was also demonstrated that vitamin D_3 plays a positive effect on the remyelination process by endogenous progenitor cells and support its possible therapeutic effects in the context of demyelinating disease like MS [16]. Vitamin D has been shown to have a direct effect on neural stem cell proliferation, survival and neuron/oligodendrocyte differentiation, thus representing a novel mechanism underlying its remyelinating and neuroprotective effect in MS/EAE therapy [30]. A deficiency in vitamin D resulted in an increased susceptibility to EAE and vitamin D_3 or its analogs might potentially be important for treatment of MS [3]. It was demonstrated that vitamin D_3 acts on myelination by means of the activation of several myelin-associated genes [4]. The data reveal a role for vitamin D in the regenerative component of demyelinating disease and identify a new target for remyelination medicines [7].

Although many studies have demonstrated the positive role of vitamin D_3 in the remyelination process of the EAE mice model, but the mechanism underly-

ing its effect is not clearly understood. $1,25(OH)_2D_3$ has been shown to be potentially effective to block the development of autoimmune diseases [5].

It was suggested that CNPase may play an important role as a putative anti-inflammatory gene both in normal and injured brain and it might be a potential target self-antigen in MS [25,35]. It was suggested that the disturbances in CNPase activity may contribute, in some extent, to the changes in myelin morphology and CNPase may play a role in cellular processes requiring membrane structural reorganization [6,19].

Myelin oligodendrocyte glycoprotein is identified by monoclonal antibody 8-18C5. Myelin oligodendrocyte glycoprotein is localized on the surface of myelin and oligodendrocyte processes and its expression level may be modulated by the presence of compact myelin and/or MBP in the myelin sheath [24]. Several studies have shown a role of antibodies against MOG in the pathogenesis of MS [10]. Growth factors including insulin like growth factor were shown to be important in repair processes of demyelination [12]. Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) have been shown to have an important role in the process of remyelination by increasing Opalin (34 KDa) and MOG expression [23,29]. It is suggested that the activation of MOG transcription depends more on an intrinsic oligodendroglial maturation program of myelination. MOG mRNA expression is restricted to CNS tissue, and peak expression occurs during active myelination [13]. It was demonstrated that NKT cells are important mediators of $1,25D_3$-induced protection from EAE in mice and NKT cell-derived IL-4 may be an important factor in providing this protection [34]. It was shown that vitamin D receptor signaling regulates neuromuscular maintenance and enhances locomotive ability after physical exercise and Schwann cells and the neuromuscular junction are targets of vitamin D_3 signaling in locomotive ability [28].

The role of vitamin D_3 in the process of remyelination has been demonstrated [26]. In our knowledge this study is the first one to demonstrate the effect of vitamin D_3 on CNPase and MOG expression in the CNS of the cuprizone-induced mice model of MS. In this study we show that administration of vitamin D significantly increases CNPase and MOG expression in cuprizone induced mice cerebral cortex. As CNPase is a molecular marker for myelin forming cells, the increased CNPase expression in the cerebral cortex of a vitamin D-injected mouse may be due to increased differentiation of oligodendrocyte progenitor cells to mature oligodendrocyte. We have also shown that vitamin D increases MOG expression in the cerebral cortex which indicates the role of vitamin D in myelin formation.

The results of this study could have been anticipated with some certainty given the findings of other studies that have investigated the role of vitamin D in MS. It is also concluded that vitamin D_3 may have an important role in the process of remyelination by increasing CNPase and MOG expression.

Acknowledgements

We would like to thank the Islamic Azad University of Tonekabon branch, Tonekabon, Iran, for the financial support.

Disclosure

Authors report no conflict of interest.

References

1. Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. Ann Neurol 2004; 55: 458-468.
2. Berger T, Reindl M. Lack of association between antimyelin antibodies and progression to multiple sclerosis. N Engl J Med 2007; 356: 1888-1889.
3. Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci U S A 1996; 93: 7861-7864.
4. Chabas JF, Stephan D, Marqueste T, Garcia S, Lavaut MN, Nguyen C, Legre R, Khrestchatisky M, Decherchi P, Feron F. Cholecalciferol (vitamin D3) improves myelination and recovery after nerve injury. PLoS One 2013; 8: e65034.
5. Chiuso-Minicucci F, Ishikawa LL, Mimura LA, Fraga-Silva TF, França TG, Zorzella-Pezavento SF, Marques C, Ikoma MR, Sartori A. Treatment with Vitamin D/MOG Association Suppresses Experimental Autoimmune Encephalomyelitis. PLoS One 2015; 10: e0125836.
6. Dabrowska-Bouta B, Sulkowski G, Walski M, Struzyńska L, Lenkiewicz A, Rafałowska U. Acute lead intoxication in vivo affects myelin membrane morphology and CNPase activity. Exp Toxicol Pathol 2000; 52: 257-263.
7. de la Fuente AG, Errea O, van Wijngaarden P, Gonzalez GA, Kerninon C, Jarjour AA, Lewis HJ, Jones CA, Nait-Oumesmar B, Zhao C, Huang JK, Ffrench-Constant C, Franklin RJ. Vitamin D receptor-retinoid X receptor heterodimer signaling regulates oligodendrocyte progenitor cell differentiation. J Cell Biol 2015; 211: 975-985.
8. Disanto G, Chaplin G, Morahan JM, Giovannoni G, Hyppönen E, Ebers GC, et al. Month of birth, vitamin D and risk

of immune-mediated disease: a case control study. BMC Med 2012; 10: 69.
9. Domańska-Janik K, Sypecka J, Taraszewska A. Immunohistochemical study of myelin-specific proteins in pt rabbits. Folia Neuropathol 1997; 35: 1-7.
10. Egg R, Reindl M, Deisenhammer F, Linington C, Berger T. Anti-MOG and anti-MBP antibody subclasses in multiple sclerosis. Mult Scler 2001; 7: 285-289.
11. Fontoura P, Steinman L, Miller A. Emerging therapeutic targets in multiple sclerosis. Curr Opin Neurol 2006; 19: 260-266.
12. Fushimi S, Shirabe T. Expression of insulin-like growth factors in remyelination following ethidium bromide-induced demyelination in the mouse spinal cord. Neuropathology 2004; 24: 208-218.
13. Gardinier MV, Matthieu JM. Cloning and cDNA sequence analysis of myelin/oligodendrocyte glycoprotein: a novel member of the immunoglobulin gene superfamily. Schweiz Arch Neurol Psychiatr 1993; 144: 201-207.
14. Genain CP, Nguyen MH, Letvin NL, Pearl R, Davis RL, Adelman M, Lees MB, Linington C, Hauser SL. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. J Clin Invest 1995; 96: 2966-2974.
15. Gomez-Lira M, Moretto G, Bonamini D, Benedetti MD, Pignatti PF, Rizzuto N, Salviati A. Myelin oligodendrocyte glycoprotein polymorphisms and multiple sclerosis. J Neuroimmunol 2002; 133: 241-243.
16. Goudarzvand M, Javan M, Mirnajafi-Zadeh J, Mozafari S, Tiraihi T. Vitamins E and D3 attenuate demyelination and potentiate remyelination processes of hippocampal formation of rats following local injection of ethidium bromide. Cell Mol Neurobiol 2010; 30: 289-299.
17. Hanwell HE, Banwell B. Assessment of evidence for a protective role of vitamin D in multiple sclerosis. Biochim Biophys Acta 2011; 1812: 202-212.
18. Hauser SL, Oksenberg JR. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. Neuron 2006; 52: 61-76.
19. Heath AR, Hindman HM. CNPase activity in the vertebrate retina, retinal pigmented epithelium, and choroid. J Exp Zool 1986; 238: 183-191.
20. Iglesias A, Bauer J, Litzenburger T, Schubart A, Linington C. T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. Glia 2001; 36: 220-234.
21. Jelinek GA, Marck CH, Weiland TJ, Pereira N, van der Meer DM, Hadgkiss EJ. Latitude, sun exposure and vitamin D supplementation: associations with quality of life and disease outcomes in a large international cohort of people with multiple sclerosis. BMC Neurol 2015; 15: 132.
22. Kipp M, Clarner T, Dang J, Copray S, Beyer C. The cuprizone animal model: new insights into an old story. Acta Neuropathol 2009; 118: 723-736.
23. Mashayekhi F, Hadiyan SP, Salehi Z. Administration of leukemia inhibitory factor increases Opalin and myelin oligodendrocyte glycoprotein expression in the cerebral cortex in a cuprizone-induced model of demyelination. Folia Neuropathol 2015; 53: 147-152.
24. Matthieu JM, Amiguet P. Myelin/oligodendrocyte glycoprotein expression during development in normal and myelin-deficient mice. Dev Neurosci 1990; 12: 293-302.
25. Muraro PA, Kalbus M, Afshar G, McFarland HF, Martin R. T cell response to 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in multiple sclerosis patients. J Neuroimmunol 2002; 130: 233-242.
26. Nystad AE, Wergeland S, Aksnes L, Myhr KM, Bø L, Torkildsen O. Effect of high-dose 1.25 dihydroxyvitamin D3 on remyelination in the cuprizone model. APMIS 2014; 122: 1178-1186.
27. Peirce TR, Bray NJ, Williams NM, Norton N, Moskvina V, Preece A, Haroutunian V, Buxbaum JD, Owen MJ, O'Donovan MC. Convergent evidence for 2',3'-cyclic nucleotide 3'-phosphodiesterase as a possible susceptibility gene for schizophrenia. Arch Gen Psychiatry 2006; 63: 18-24.
28. Sakai S, Suzuki M, Tashiro Y, Tanaka K, Takeda S, Aizawa K, Hirata M, Yogo K, Endo K. Vitamin D receptor signaling enhances locomotive ability in mice. J Bone Miner Res 2015; 30: 128-136.
29. Salehi Z, Hadiyan SP, Navidi R. Ciliary neurotrophic factor role in myelin oligodendrocyte glycoprotein expression in Cuprizone-induced multiple sclerosis mice. Cell Mol Neurobiol 2013; 33: 531-535.
30. Shirazi HA, Rasouli J, Ciric B, Rostami A, Zhang GX. 1,25-Dihydroxyvitamin D3 enhances neural stem cell proliferation and oligodendrocyte differentiation. Exp Mol Pathol 2015; 98: 240-245.
31. Soleimani M, Jameie SB, Mehdizadeh M, Keradi M, Masoumipoor M, Mehrabi S. Vitamin D3 influence the Th1/Th2 ratio in C57BL/6 induced model of experimental autoimmune encephalomyelitis. Iran J Basic Med Sci 2014; 17: 785-792.
32. Spach KM, Hayes CE. Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. J Immunol 2005; 175: 4119-4126.
33. Verway M, Bouttier M, Wang TT, Carrier M, Calderon M, An BS, et al. Vitamin D induces interleukin-1β expression: paracrine macrophage epithelial signaling controls M. tuberculosis infection. PLoS Pathog 2013; 9: e1003407.
34. Waddell A, Zhao J, Cantorna MT. NKT cells can help mediate the protective effects of 1,25-dihydroxyvitamin D3 in experimental autoimmune encephalomyelitis in mice. Int Immunol 2015; 27: 237-244.
35. Yang L, Kan EM, Lu J, Wu C, Ling EA. Expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and its roles in activated microglia in vivo and in vitro. J Neuroinflammation 2014; 11: 148.
36. Yeshokumar AK, Saylor D, Kornberg MD, Mowry EM. Evidence for the Importance of Vitamin D Status in Neurologic Conditions. Curr Treat Options Neurol 2015; 17: 51.

Original paper

Folia Neuropathologica

Protective effects of peel and seed extracts of *Citrus aurantium* on glutamate-induced cytotoxicity in PC12 cell line

Azar Hosseini[1], Hamid Reza Sadeghnia[1,2,3], Arezoo Rajabian[2]

[1]Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, [2]Department of Pharmacology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, [3]Neurocognitive Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Folia Neuropathol 2016; 54 (3): 265-272 DOI: 10.5114/fn.2016.62536

Abstract

Oxidative stress and apoptosis contribute to neuronal degeneration in many neurodegenerative diseases such as Alzheimer's disease. Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS) and is considered responsible for the pathogenesis of many neurological disorders. Reactive oxygen species (ROS) production is thought to be involved in glutamate-induced apoptosis process. In this study, the neuroprotective effects of Citrus aurantium in the glutamate-induced rat's adrenal pheochromocytoma cell line (PC12 cells) were investigated. The cell viability and apoptotic cell death were measured using MTT and propidium iodine (PI)-staining methods, respectively. In addition, intracellular ROS and malondialdehyde (MDA) levels were determined by fluorometric methods. The results showed that glutamate cytotoxicity in PC12 cells was accompanied by an increment of MDA content, ROS generation, and apoptotic induction. However, pretreatment with peel and seed extracts of C. aurantium significantly reduced MDA content, ROS generation, and apoptotic cells. All these findings indicated that C. aurantium protected PC12 cells against glutamate-induced apoptosis by inhibiting ROS production. Therefore, the present study supports that C. aurantium extracts possess neuroprotective effects against glutamate-induced toxicity in PC12 cell line. The protective effect of C. aurantium might be attributed to its antioxidant properties.

Key words: *apoptosis, Citrus aurantium, glutamate cytotoxicity, neuroprotection, PC12.*

Introduction

Glutamate is the most excitatory neurotransmitter in the brain. It is an important neurotransmitter for learning and memory in the central nervous system [32]. Excessive amounts of glutamate may act as a potent neurotoxin by activating the proteolytic enzymes [39]. Naturally, glutamate is released into the synaptic cleft and binds to glutamate receptors resulting in the propagation of an action potential [22]. However, increased amounts of glutamate in the synaptic cleft can lead to neurotoxicity. It has been reported that glutamate increases in patients who suffer from Alzheimer's disease [11]. PC12 cell line is derived from a pheochromocytoma of rat adrenal medulla. This cell model is also widely applied to study cellular glutamate toxicity [38]. Therefore, it is an appropriate model to investigate the protective effect of the compounds on glutamate toxicity. *Citrus aurantium* L. (*Rutaceae*), also called

Communicating author

Arezoo Rajabian, Department of Pharmacology, Faculty of Medicine, Mashhad University of Medical Sciences, 511 8828567 Mashhad, Iran, phone: +98 511 8828567, e-mail: rajabianar@gmail.com

Bitter orange, has been used as a traditional medicine for more than 5,000 years [5]. Citrus fruits and their products are rich sources of health-promoting constituents and are widely consumed around the world [5]. They increase serum antioxidant capacity against lipid peroxidation [3] and reduce the elderly oxidative stress. Citrus flavonoids have been shown to prevent oxidative stress, attenuate inflammation and exhibit anti-tumor [31,33] and anti-atherosclerosis properties [27]. In addition, citrus fruits serve as a supplement of chemotherapy [26], diabetic health food [2], and neuroprotection [15]. Furthermore, the aqueous extract from the leaves of the *C. aurantium* have anticonvulsant properties [34]. Considering the antioxidant properties, this research focuses on protective effects of peel and seed extracts of *C. aurantium* against glutamate toxicity in PC12 cells.

Material and methods

Reagents and chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), thiobarbituric acid (TBA), 2,7-dichlorofluorescin diacetate (DCFH-DA), propidium iodide (PI), sodium citrate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco's Modified Eagles Medium (DMEM), penicillin-streptomycin, and fetal bovine serum were purchased from Gibco (Carlsbad, CA, USA). Trichloroacetic acid (TCA), malondialdehyde bis(dimethyl acetal) (MDA) and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). PC12 cells were obtained from Pasteur Institute (Tehran, Iran).

Preparation of the extracts

Peels and seeds of *C. aurantium* were washed, dried, and crushed to powder with an electric micronizer. The peels and seeds were extracted separately in a Soxhlet extractor with ethanol (70%) and the obtained extract was then dried and kept at –20°C until use. The obtained extracts were dissolved in dimethyl sulfoxide and then subjected to cytotoxic and apoptosis assays.

Cell culture and treatment

The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 100 Units/ml penicillin and 100 µg/ml streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT/ROS and MDA assays, respectively. For apoptosis assay, the cells were seeded at 100,000 cell/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with the extract alone (6 to 200 µg/ml) for 2 h and then incubation was continued in the presence of the extract with or without 8 mM glutamate for 24 h.

Cell viability assay

Cell viability was determined using a modified MTT assay as described previously [13]. Briefly, MTT solution in phosphate-buffered saline (5 mg/ml) was added to each well at a final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance of 570 and 620 nm (background) was measured using a StatFAX303 plate reader.

Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring MDA, which is the end product of lipid peroxidation [13]. At the end of incubation, the cells were scraped and centrifuged for 30 min. Then, 400 µl of TCA (15%) and 800 µl of TBA (0.7%) were added to 500 µl of cell samples. The mixture was vortexed and heated for 40 min in a boiling water bath. Then, 200 µl of the sample was transferred to a 96-well plate and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

Measurement of reactive oxygen species

The intracellular ROS level was evaluated using a fluorescent probe, DCFH-DA. At the end of incubation, the cells were treated (30 min) with DCFH-DA (10 µM) at 4°C in the dark. Then, the fluorescence intensity was detected with excitation/emission of 485/530 nm. The experiment was performed in triplicate.

Propidium iodine staining

Apoptotic cells were detected using PI staining of small DNA fragments followed by flow cytometry. In the cells exposed to a hypotonic phosphate-citrate buffer containing PI, a quantitative DNA-binding dye, a sub-G1 peak is reflective of the DNA fragmentation. Apoptotic cells that had lost DNA will take up less stain and appear on the left side of the G1 peak in the histogram. Briefly, the cells were seeded

in a 24-well plate and treated according to the mentioned protocol. Floating and adherent cells were then harvested and incubated at 4°C overnight in the dark with 750 µl of a hypotonic buffer (50 µg/ml PI in 0.1% sodium citrate with 0.1% Triton X-100). Next, flow cytometry was carried out using a FACScan flow cytometer (Becton Dickinson). A total of 10^4 events were achieved with FACS.

Statistics

All the data were expressed as mean ± SEM. Statistical analyses were performed using Prism 6 software. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer post hoc test for comparison between groups. The differences were considered significant at $p < 0.05$.

Results

The effect of the peel and seed extracts on the cell viability

To study the possible toxic effects of the extracts, PC12 cells were incubated with different concentrations of the extracts (12-200 µg/ml), and the viability was determined 24 h after the treatment. No significant toxic effect on the cell viability was seen subsequent to the treatment with the extracts.

Incubation with glutamate significantly decreased cell viability to 43.7 ± 1.4% of control ($p < 0.001$). As shown in Figure 1A, the treatment with the peel extract increased cell viability following glutamate insult at a concentration of 25-200 µg/ml, in a concentration-dependent manner (25 µg/ml, 61.36 ± 2%, $p < 0.05$; 50 µg/ml, 64.62 ± 1%, $p < 0.01$; 100 µg/ml, 69.62 ± 1.9%, $p < 0.001$; 200 µg/ml, 80 ± 3.7%, $p < 0.001$). Moreover, the results showed that the seed extract increased cell viability at the concentrations of 50-200 µg/ml (50 µg/ml, 63 ± 1.6%, $p < 0.01$; 100 µg/ml, 65 ± 0.98%, $p < 0.001$; 200 µg/ml, 70 ± 1.9%, $p < 0.001$) (Fig. 1B).

The effects of the peel and seed extracts on ROS content

As expected, glutamate caused a significant increase in the level of ROS in PC12 cells (260 ± 7.8% of control, $p < 0.001$). The peel extract at concentrations of 50, 100 and 200 µg/ml was able to decrease the intracellular ROS level to 219.8 ± 10.37% ($p < 0.05$), 206 ± 7.8% ($p < 0.01$) and 152 ± 6.7% ($p < 0.001$) of control, respectively (Fig. 2A). The seed extract decreased the ROS level at concentrations of 100 µg/ml (212 ± 5.2%, $p < 0.01$) and 200 µg/ml (189 ± 7.6%, $p < 0.001$) (Fig. 2B).

The effect of the peel and seed extracts on lipid peroxidation

The level of lipid peroxidation was evaluated by measuring the level of MDA, which is the end product of lipid peroxidation. As shown in Figure 3, exposure of the cells to glutamate resulted in a significant increase of MDA level (269 ± 7.5%, $p < 0.001$) as compared to control cells cultured in the absence of glutamate (100 ± 4.5%). The content of MDA signifi-

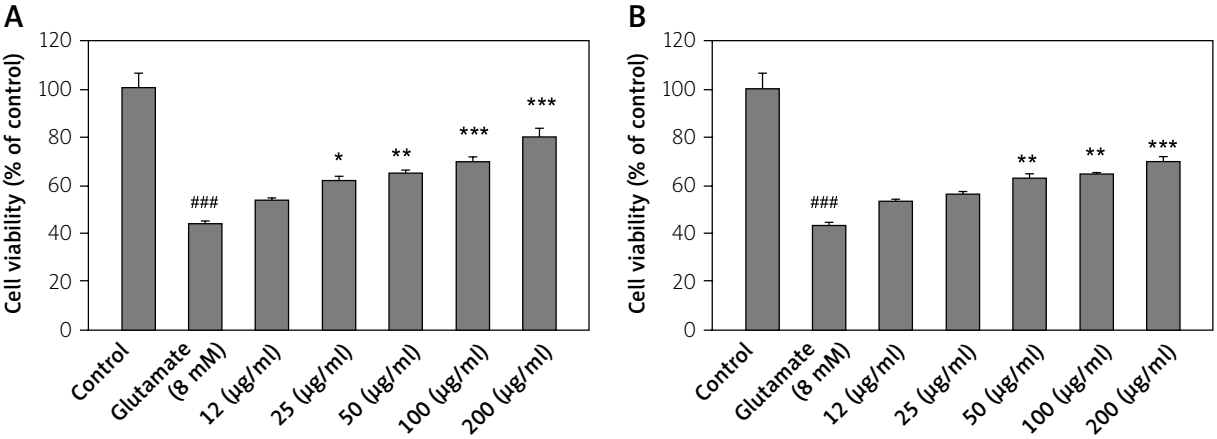


Fig. 1. Effect of *Citrus aurantium* extracts on cell viability of PC12 cells. The cells were pretreated with different concentrations of the peel (**A**) and seed (**B**) extracts for 2 h prior to glutamate exposure. Viability was quantitated using MTT assay. Results are mean ± SEM ($n = 3$). $^{###}p < 0.001$ vs. control, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. glutamate.

cantly decreased in the cells pretreated with 50 µg/ml (222.4 ± 11.3%, $p < 0.001$), 100 µg/ml (194 ± 6.86%, $p < 0.001$) and 200 µg/ml (147 ± 8.4%, $p < 0.001$) of the skin extract (Fig. 3A). The seed extract reduced the level of MDA at 100 µg/ml (206 ± 7.5%, $p < 0.05$) and 200 µg/ml (160 ± 8.4%, $p < 0.001$) (Fig. 3B).

The effects of the peel and seed extracts on apoptotic cells

Apoptosis in PC12 cell line was detected with flow cytometry using PI staining. Cells were pretreated for 2 h with various concentrations of the *C. aurantium* and exposed to glutamate for 24 h. Analysis of the sub-G1 peak in flow cytometry histograms revealed the induction of apoptosis in the cells treated with glutamate ($p < 0.001$). As shown in Figure 4A and 4B, the peel and the seed extracts of *C. aurantium* decreased apoptotic induction significantly at the doses of 50, 100 and 200 µg/ml, respectively.

Discussion

The present study is the first report to evaluate neuroprotective effects of peel and seed extracts of *C. aurantium* against apoptotic cell death induced by glutamate toxicity in PC12 cells. After treating PC12 cells with 8 mM glutamate, a significant decrease in the cell viability and increase in the number of apoptotic cells, ROS and MDA contents were seen, confirming its neurotoxic effect on PC12 cells. PC12 cells were used because they constitute a widely-

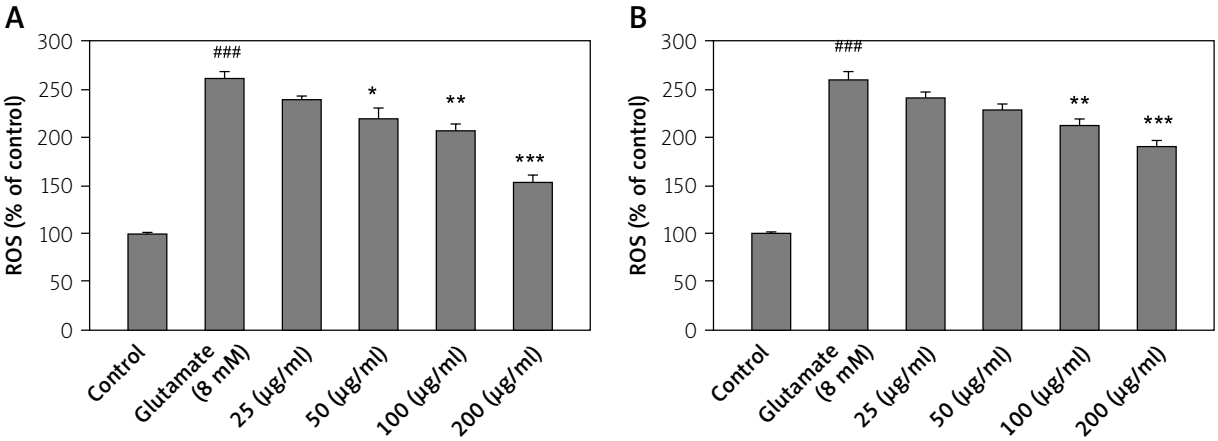


Fig. 2. Effect of *Citrus aurantium* extracts on ROS generation. The cells were pretreated with different concentrations of the peel (**A**) and seed (**B**) extracts for 2 h prior to glutamate exposure. Results are mean ± SEM ($n = 3$). $^{###}p < 0.001$ vs. control, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. glutamate.

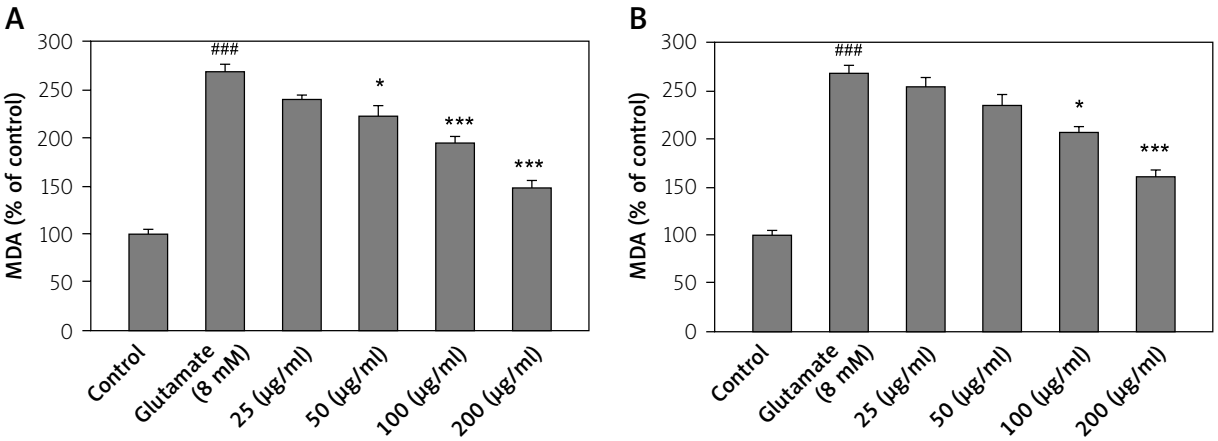


Fig. 3. Effect of *Citrus aurantium* extracts on MDA production. The cells were pretreated with different concentrations of the peel (**A**) and seed (**B**) extracts for 2 h then prior to glutamate exposure. Results are mean ± SEM ($n = 3$). $^{###}p < 0.001$ vs. control, $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. glutamate.

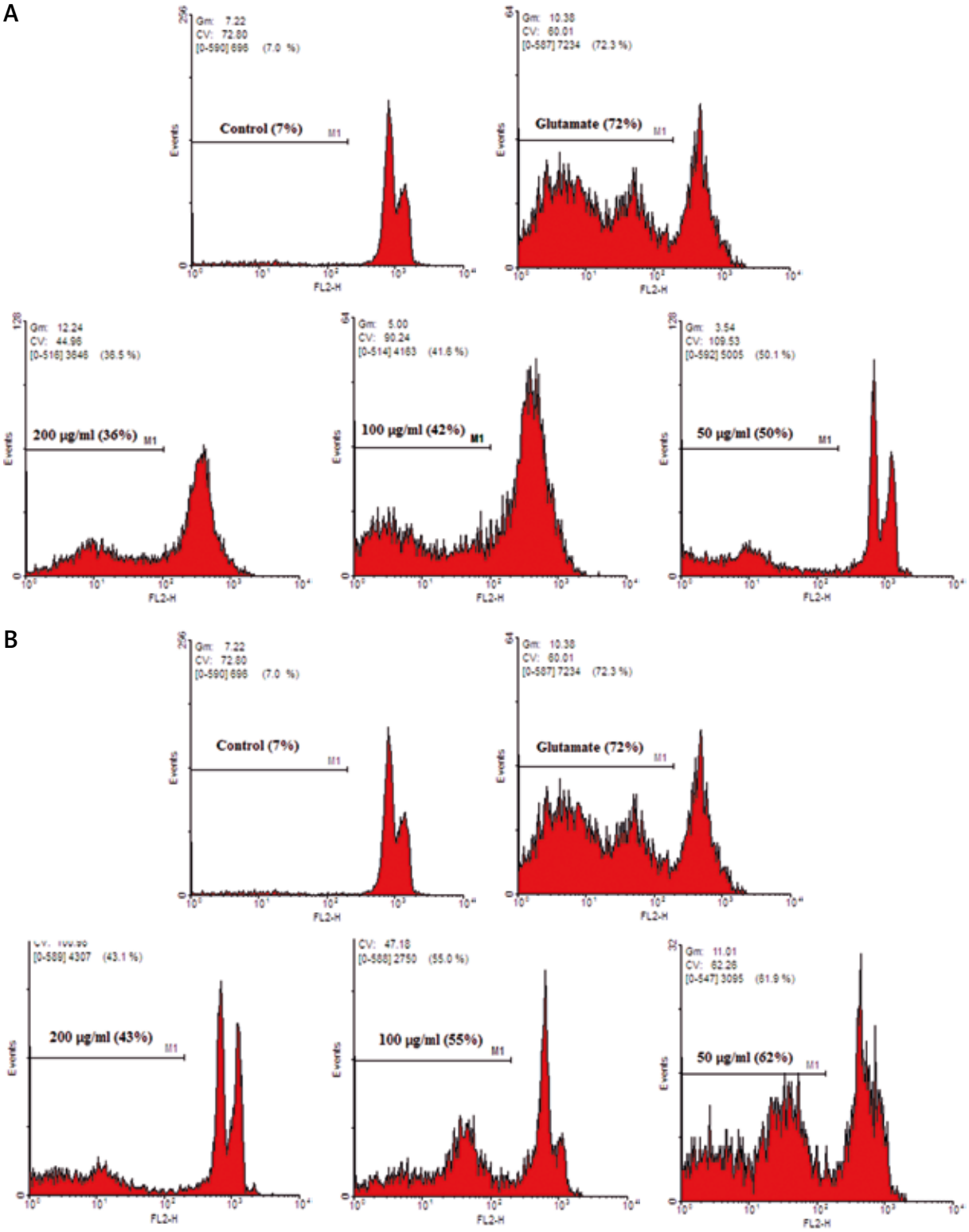


Fig. 4. The effects of the peel (**A**) and seed (**B**) extracts of *Citrus aurantium* extracts on apoptosis in PC12 cells using propidium iodine staining and flow cytometry.

used neuronal model system [17]. PC12 cells are more sensitive to glutamate injury; therefore, this cell model is also widely applied to study cellular glutamate toxicity [38]. Results showed that the pretreatment with *C. aurantium* peel and seed extracts can decrease glutamate-toxicity with different potencies. Our findings indicated that the peel extract has a more protective effect than the seed extract. Glutamate, a major excitatory neurotransmitter, plays an important role in synaptic transmission, formation of neuronal circuit, and neuronal development in the nervous system [37]. The elevated level of glutamate due to excess release or/and uptake disorder led to induce extensive neuronal damage and cell loss in brain tissue. Glutamate cytotoxicity has been associated with the activation of glutamate receptors and non-receptor-mediated oxidative glutamate toxicity [4,35]. Recent studies have shown that glutamate-induced cytotoxicity could be mediated by oxidative stress, depletion of GSH, and down regulation of SOD activity that leads to apoptosis [18]. Reactive oxygen species are typically defined as molecules or ions formed by the incomplete one-electron reduction of oxygen. Elevated levels of ROS are well-known etiological factors associated with oxidative stress leading to cell death via apoptosis in a variety of cell types [20,36], and such effects can be blocked or delayed by a wide variety of antioxidants [30]. Such antioxidants are reported to scavenge free radicals by raising the levels of endogenous antioxidant defense systems such as glutathione peroxidase and glutathione reductase [8,28]. Consequently, pretreatment of the cells with *C. aurantium* extracts prior to glutamate exposure resulted in a significant decrease in ROS and MDA content as well as apoptotic cells. *Citrus aurantium* peel contains citral, limonene, and several citrus bioflavonoids, including hesperidin, neohesperidin, naringin, and rutin [29]. These compounds have been attributed with a range of properties. For example, naringin is believed to cross the blood brain barrier [1,40] and to have antioxidant, anti-inflammatory, anti-hypercholesterolemic, anti-hypertensive, neuroprotective [9,6,16], and anticonvulsant properties. It also increases the seizure latency to kainic acid administration [9]. Naringenin and hesperetin exhibit antiatherogenic properties by activating the peroxisome proliferator-activated receptor (PPAR) and up-regulating adiponectin expression [23]. Hesperidin, neohesperidin, and neohesperidin dihydrochalcone, the most abundant species detected, are attributed antioxidant [14,19], anti-inflammatory [10,12], and vasopressive and antiplatelet properties [24] and may be responsible for the anticonvulsant properties of the *C. aurantium* extract. However, the peels and seeds of citrus fruits are rich in nutrients and contain many phytochemicals with strong potential to be used in drug production or as food supplements [7,21,25]. Some studies showed that *C. aurantium* has direct antioxidant activity. For example, Karimi *et al*. (2012) reported that *C. aurantium* extract scavenges free radicals and exhibits ferric reducing potential, with values of 55.3% and 51.7%, respectively [16]. The obtained results from pretreatment of cells with *C. aurantium* extracts demonstrated increased antioxidant capacity of cells prior to exposure to glutamate may be involved in cytoprotective properties of the extracts. In consistent with our results, other investigations also showed antioxidative properties of its bioactive constituents [6,9].

In conclusion, our study demonstrated that *C. aurantium* has an apparently neuroprotective effect against glutamate oxidative damage through its antioxidant activity. However, further studies are required to elucidate its molecular mechanism before the clinical therapeutic application of these extracts.

Acknowledgements

This work was supported by the Office of the Vice Chancellor for Research and Technology of Mashhad University of Medical Sciences. The authors are gratefully acknowledged for this financial support.

Disclosure

Authors report no conflict of interest.

References

1. Ameer B, Weintraub RA, Johnson JV, Yost RA, Rouseff RL. Flavanone absorption after naringin, hesperidin, and citrus administration. Clin Pharmacol Ther 1996; 60: 34-40.
2. Aruoma OI, Landes B, Ramful-Baboolall D, Bourdon E, Neergheen-Bhujun V, Wagner KH, Bahorun T. Functional benefits of citrus fruits in the management of diabetes. Prev Med 2012; 54: S12-S16.
3. Assini JM, Mulvihill EE, Sutherland BG, Telford DE, Sawyez CG, Felder SL, Chhoker S, Edwards JY, Gros R, Huff MW. Naringenin prevents cholesterol-induced systemic inflammation, metabolic dysregulation, and atherosclerosis in Ldlr/mice. J Lipid Res 2013; 54:711-724.

4. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 1995; 92: 7162-7166.
5. Castleman M. The Healing Herbs: The Ultimate Guide to the Curative Power of Nature's Medicines. Bantam Books, New York 1995.
6. Chanet A, Milenkovic D, Manach C, Mazur A, Morand C. Citrus flavanones: what is their role in cardiovascular protection? J Agric Food Chem 2012; 60: 8809-8822.
7. Chede PS. Phytochemical analysis of *Citrus sinensis* peel. Int J Pharm Bio Sci 2013; 4: 339-343.
8. Gao Y, Zhang HW, Qiao HL, Wang W, Chang JB. Protective effect of 3-butyl-6-bromo-1(3H)-isobenzofuranone on hydrogen peroxide-induced damage in PC12 cells. Brain Res 2010; 1358: 239-247.
9. Golechha M, Chaudhry U, Bhatia J, Saluja D, Arya DS. Naringin protects against kainic acid-induced status epilepticus in rats: evidence for an antioxidant, anti-inflammatory and neuroprotective intervention. Biol Pharm Bull 2011; 34: 360-365.
10. Hamdan DI, Mahmoud MF, Wink M, El-Shazly AM. Effect of hesperidin and neohesperidin from bittersweet orange (*Citrus aurantium* var. bigaradia) peel on indomethacin-induced peptic ulcers in rats. Environ Toxicol Pharmacol 2014; 37: 907-915.
11. Hazell AS, Itzhak Y, Liu H, Norenberg MD. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in cultured astrocytes. J Neurochem 1997; 68: 2216-2219.
12. Ho SC, Kuo CT. Hesperidin, nobiletin, and tangeretin are collectively responsible for the anti-neuroinflammatory capacity of tangerine peel (Citri reticulatae pericarpium). Food Chem Toxicol 2014; 71: 176-182.
13. Hosseini A, Shafiee-Nick R, Mousavi SH. Combination of Nigella sativa with Glycyrrhiza glabra and Zingiber officinale augments their protective effects on doxorubicin-induced toxicity in h9c2 Cells. Iran J Basic Med Sci 2014; 17: 993-1000.
14. Hu L, Li L, Xu D, Xia X, Pi R, Xu D, Wang W, Du H, Song E, Song Y. Protective effects of neohesperidin dihydrochalcone against carbon tetrachloride-induced oxidative damage in vivo and in vitro. Chem Biol Interact 2014; 213: 51-59.
15. Hwang SL, Shih PH, Yen GC. Neuroprotective effects of citrus flavonoids. J Agric Food Chem 2012; 60: 877-885.
16. Karimi E, Oskoueian E, Hendra R, Oskoueian A, Jaafar HZE. Phenolic compounds characterization and biological activities of *Citrus aurantium* bloom. Molecules 2012; 17: 1203-1218.
17. Kazmierczak A, Strosznajder JB, Adamczyk A. α-Synuclein enhances secretion and toxicity of amyloid beta Peptides in PC12 cells. Neurochem Int 2008; 53: 263-269.
18. Kritis AA, Stamoula EG, Paniskaki KA, Vavilis TD. Researching glutamate-induced cytotoxicity in different cell lines: a comparative/collective analysis/study. Front Cell Neurosci 2015; 9: 91.
19. Kumar A, Lalitha S, Mishra J. Possible nitric oxide mechanism in the protective effect of hesperidin against pentylenetetrazole (PTZ)-induced kindling and associated cognitive dysfunction in mice. Epilepsy Behav 2013; 29: 103-111.
20. Lan AP, Xiao LC, Yang ZL, Yang CT, Wang XY, Chen PX, Gu MF, Feng JQ. Interaction between ROS and p38MAPK contributes to chemical hypoxia-induced injuries in PC12 cells. Mol Med Rep 2012; 5: 250-255.
21. Lawal D, Bala JA, Aliyu SY, Huguma MA. Phytochemical screening and in vitro anti-bacterial studies of the ethanolic extract of *Citrus Sinensis* (Linn.) peel against some clinical bacterial isolates. International Journal of Innovation and Applied Studies 2013; 2: 138-145.
22. Li N, Liu B, Dluzen DE, Jin Y. Protective effects of ginsenoside Rg2 against glutamate-induced neurotoxicity in PC12 cells. J Ethnopharmacol 2007; 111: 458-463.
23. Liu L, Shan S, Zhang K, Ning Z, Lu X, Cheng, Y. Naringenin and hesperetin, two avonoids derived from *Citrus aurantium* up-regulate transcription of adiponectin. Phyther Res 2008; 1403: 1400-1403.
24. Majumdar S, Srirangam R. Solubility, stability, physicochemical characteristics and in vitro ocular tissue permeability of hesperidin: a natural bioflavonoid. Pharm Res 2009; 26: 1217-1225.
25. Mathew B, Jatawa SK, Tiwaari A. Phytochemical analysis of *Citrus limonum* pulp and peel. Int J Pharm Pharm Sci 2012; 4: 269-371.
26. Meiyanto E, Hermawan A, Anindyajati A. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pac J Cancer Prev 2012; 13: 427-436.
27. Mulvihill EE, Huff MW. Citrus flavonoids and the prevention of atherosclerosis. Cardiovasc Hematol Disord Drug Targets 2012; 12: 84-91.
28. Nichols JA, Katiyar SK. Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. Arch Dermatol Res 2010; 302: 71-83.
29. Okwu DE. Citrus fruits: a rich source of phytochemicals and their roles in human health. Int J Chem Sci 2008; 6: 451-471.
30. Ozkan OV, Yuzbasioglu MF, Ciralik H, Kurutas EB, Yonden Z, Aydin M, Bulbuloglu E, Semerci E, Goksu M, Atli Y, Bakan V, Duran N. Resveratrol, a natural antioxidant, attenuates intestinal ischemia/reperfusion injury in rats. Tohoku J Exp Med 2009; 218: 251-258.
31. Park E, Pezzuto JM. Flavonoids in cancer prevention. Anticancer Agents Med Chem 2012; 12: 836-851.
32. Penugonda S, Mare S, Lutz P, Banks WA, Ercal N. Potentiation of lead-induced cell death in PC12 cells by glutamate: protection by N-acetylcysteine amide (NACA), a novel thiol antioxidant. Toxicol Appl Pharmacol 2006; 216: 197-205.
33. Romagnolo DF, Selmin OI. Flavonoids and cancer prevention: a review of the evidence. J Nutr Gerontol Geriatr 2012; 31: 206-238.
34. Rosa-Falero C, Torres-Rodríguez S, Jordán C, Licier R, Santiago Y, Toledo Z, Santiago M, Serrano K, Sosa J, Ortiz JG. *Citrus aurantium* increases seizure latency to PTZ induced seizures in zebrafish thru NMDA and mGluR's I and II. Front Pharmacol 2015; 13: 284.
35. Seyfried J, Evert BO, Rundfeldt C, Schulz JB, Kovar KA, Klockgether T, Wullner U. Flupirtine and retigabine prevent L-glutamate toxicity in rat pheochromocytoma PC12 cells. Eur J Pharmacol 2000; 400: 155-166.
36. Tao L, Li X, Zhang L, Tian J, Li X, Sun X, Li X, Jiang L, Zhang X, Chen J. Protective effect of tetrahydroxystilbene glucoside on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. PLoS One 2011; 6: e26055.

37. Walton HS, Dodd PR. Glutamate-glutamine cycling in Alzheimer's disease. Neurochem Int 2007; 50: 1052-1066.
38. Wang CJ, Hu CP, Xu KP, Yuan Q, Li FS, Zou H, Tan GS, Li YJ. Protective effect of selaginellin on glutamate-induced cytotoxicity and apoptosis in differentiated PC12 cells. Naunyn Schmiedebergs Arch Pharmacol 2010; 381: 73-81.
39. Weil ZM, Norman GJ, DeVries AC, Nelson RJ. The injured nervous system: a Darwinian perspective. Prog Neurobiol 2008; 86: 48-59.
40. Zbarsky V, Datla KP, Parkar S, Rai DK, Aruoma OI, Dexter DT. Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease. Free Radic Res 2005; 39: 1119-1125.

Original paper

Folia Neuropathologica

Warburg micro syndrome type 1 associated with peripheral neuropathy and cardiomyopathy

Dagmara Kabzińska[1], Hanna Mierzewska[2], Jan Senderek[3], Andrzej Kochański[1]

[1]Neuromuscular Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, [2]Department of Pediatric and Adolescent Neurology, Institute of Mother and Child, Warsaw, Poland, [3]Friedrich Baur Institute, Ludwig Maximilians University of Munich, Munich, Germany

Folia Neuropathol 2016; 54 (3): 273-281 DOI: 10.5114/fn.2016.62537

Abstract

The Warburg micro syndrome (WARBM) is a genetically heterogeneous syndrome linked to at least 4 loci. At the clinical level, WARBM is characterized by microcephaly, microphthalmia, microcornea, congenital cataracts, corpus callosum hypoplasia, severe mental retardation, and hypogonadism. In some families additional clinical features have been reported. The presence of uncommon clinical features (peripheral neuropathy, cardiomyopathy) may result in misdirected molecular diagnostics. Using the next generation sequencing approach (NGS), we were able to diagnose WARBM1 syndrome by detection of a new mutation within the RAB3GAP1 gene. We have detected some DNA variants which may be responsible for cardiomyopathy. We did not find any obvious pathogenic mutation within a set of genes known to be responsible for hereditary motor and sensory neuropathy (HMSN). We conclude that: (i) in clinically delineated syndromes, a classical single-gene oriented approach may be not conclusive especially in the presence of rare clinical features, (ii) peripheral neuropathy and cardiomyopathy are rare additional symptoms coexisting with WARBM1, (iii) a pleiotropic effect of a single point mutation is sufficient to be causative for WARBM1 and (iv) more WARBM-affected patients should be reported to delineate a complete phenotype.

Key words: *Warburg syndrome, whole-exome sequencing, RAB3GAP1.*

Introduction

The Warburg micro syndrome (WARBM) is a very rare genetic disorder with an unknown frequency reported in a small group of patients (less than 100) around the world.

For the first time in 1993, WARBM was reported in a consanguineous Pakistani marriage in three affected children with mental retardation [18]. In 2005, in turn, WARBM1 was linked to the 2q21.3 region, in which inactivating mutations within the *RAB3GAP1* gene were detected [2]. In recent years, access to molecular genetic analysis has made possible the delineation of the WARBM phenotype. WARBM manifests with microcephaly, microphthalmia, microcornea, congenital cataracts, *corpus callosum* hypoplasia, hypogonadism and severe mental retardation [6]. Two WARBM-affected sisters were also found to manifest peripheral neuropathy [11].

The vast majority of WARBM patients originate from consanguineous Muslim families [1,2,14].

Communicating author

Andrzej Kochański, Neuromuscular Unit, Mossakowski Medical Centre, Polish Academy of Sciences, 5 Pawińskiego St., 02-106 Warsaw, Poland, phone: + 48 22 658 45 01, e-mail: akochanski@imdik.pan.pl

To date, four genes coding for RAB-family proteins have been reported to be mutated in WARBM syndrome, i.e. the *RAB3GAP1* gene on chromosome 2q21.3 (MIM# 602536), *RAB3GAP2* (MIM# 609275) on chromosome 1q41, the *RAB18* gene (MIM# 602207) located in the 10p12.1 region and finally the *TBC1D20* gene (MIM#611663) linked to the 20p13 *locus*. At the clinical level, WARBM1-4 subtypes are indistinguishable.

To date, the analysis of WARBM-involved genes has been directed by the presence of typical clinical features. In fact, in previous studies, only a single gene was analyzed and no data from the rest of the genome were available.

A question thus arises as to whether the whole clinical manifestation of WARBM is related to a single mutation in one of the 4 RAB genes.

In the work described here, we performed for the first time a whole-exome sequencing analysis in a Polish six-generational consanguineous WARBM pedigree.

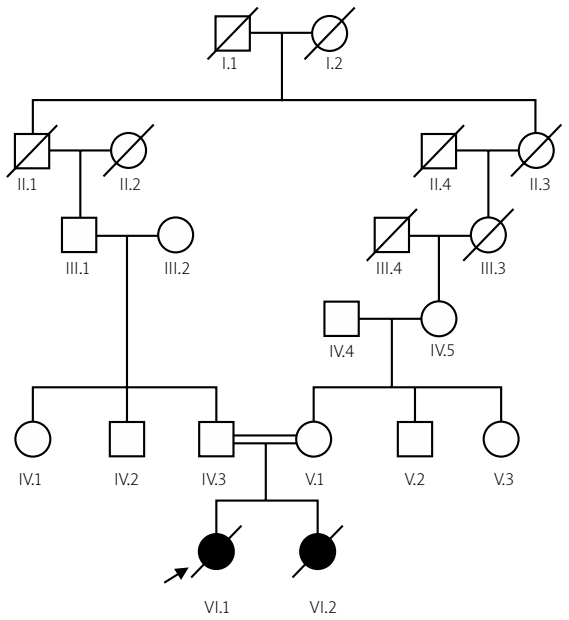


Fig. 1. Pedigree tree of the family. Note the common ancestor within the 1st generation in this consanguineous family tree. The proband is marked with an arrow. Open symbols indicate healthy males (squares) and females (circles). Filled symbols correspond to affected individuals. Deceased individuals are marked with diagonal lines. The double line indicates consanguinity in this family.

Material and methods

Case reports

The patients were two sisters who died in the second decade of life, being the only children of healthy, consanguineous (IV/V) parents (Fig. 1, Fig. 2A-C). They were born following uneventful pregnancies and deliveries. The older girl weighed 2950 g, her body length was 56 cm, OFC was 35 cm, and the Apgar score was 9 points. The younger girl weighed 2850 g, her body length was 54 cm, OFC was 32 cm, and the Apgar score was 10 points. Bilateral congenital cataract was apparent in both girls soon after birth, and a bilateral lentectomy was performed during their infancy.

The girls were severely hypotonic from early life, and their psychomotor development was delayed. At the age of a few months, infantile spasms were observed in the younger sister. Electroencephalography (EEG) showed hypsarrhythmia, so antiepileptic drugs were administered. Electroencephalography of the second sister was also severely abnormal, though no epileptic seizures were observed.

At the age of 2 years they presented with cerebellar ataxia. Limb and axial hypotonia were still observed, but a bilateral Babinski sign was also detected. Mild microcephaly was visible in both girls from early childhood.

Muscle biopsies performed at the ages of 1 and 5 years in the two sisters showed only unspecific changes in muscle fibers with mild lipid accumulation, and without any signs of myopathy or neurogenic changes (data not shown). A biochemical study of OXPHOS revealed slightly decreased activity of complex I, but blood and cerebrospinal fluid lactate were normal, both at baseline and following glucose challenge.

In the second half of the first decade of the patients' lives, mild spasticity developed, but tendon reflexes weakened progressively. Bilateral Babinski signs were still detected, and skeletal anomalies related to chronic muscle hypotonia also developed, in the form of kyphoscoliosis, thoracic deformity, and joint contractures (ulnar, iliofemoral and of the knees). Hands were dropping and planovalgus feet were to be noted. Ophthalmological examination revealed microphthalmia, microcornea and pale, atrophic optic discs. Psychomotor retardation was marked. The patients were never able to sit and walk independently. The younger girl still had epileptic seizures. The EEG of the second sister was also severely abnormal, but seizures were not observed.

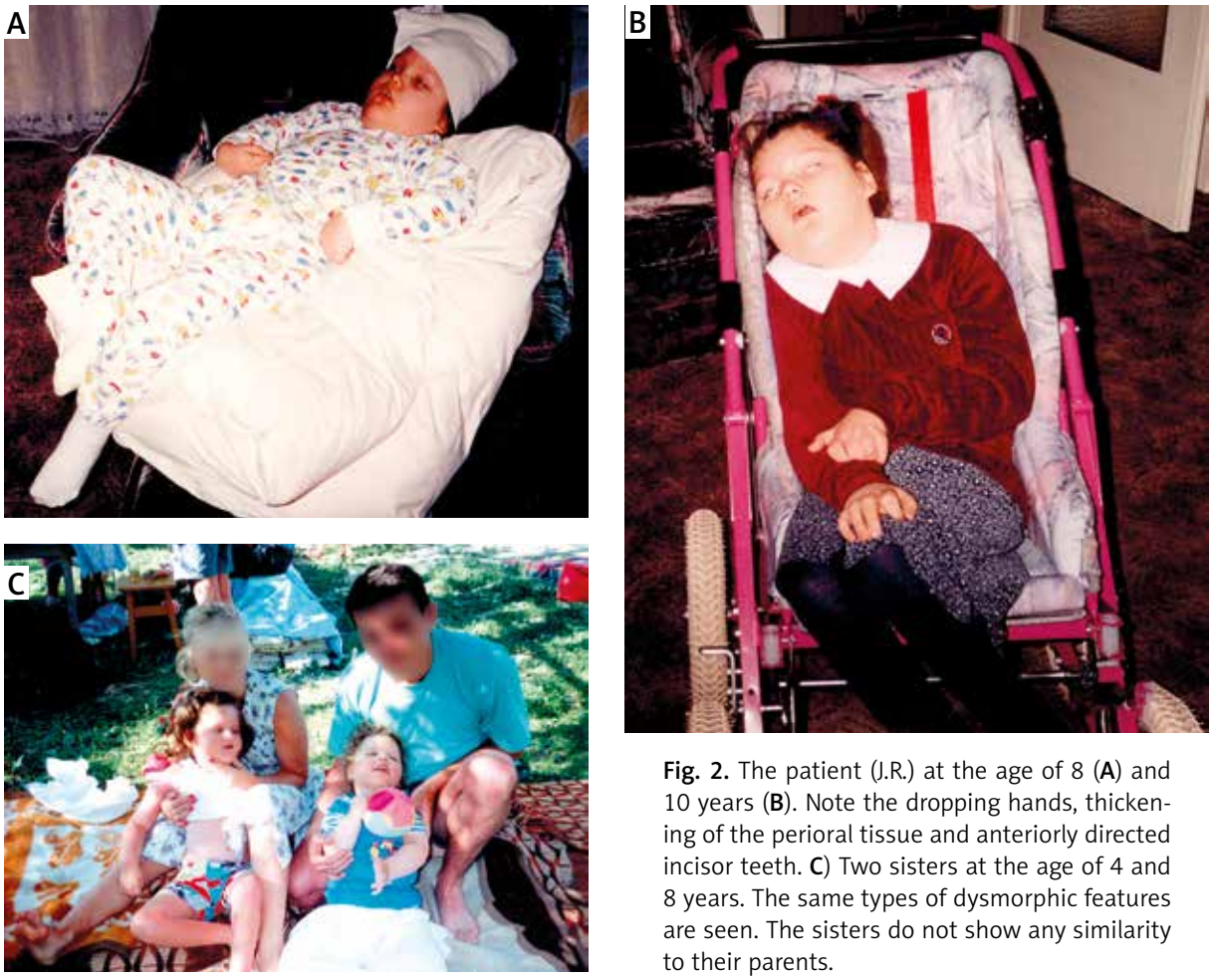


Fig. 2. The patient (J.R.) at the age of 8 (**A**) and 10 years (**B**). Note the dropping hands, thickening of the perioral tissue and anteriorly directed incisor teeth. **C**) Two sisters at the age of 4 and 8 years. The same types of dysmorphic features are seen. The sisters do not show any similarity to their parents.

Brain MRI showed slowly progressive cortico-subcortical cerebral and cerebellar atrophy as well as hypomyelination. The *corpus callosum* was thin and the brainstem was atrophic (Fig. 3). The Nerve Conduction Study (NCS) performed at 7 and 11 years, respectively, revealed progressive motor and sensory neuropathy, with demyelination and mildly expressed axonal degeneration. EMG showed neurogenic changes.

Both girls were of short stature, and deficient as regards body weight. The older girl was hypogonadal. The older girl had cardiomyopathy with left ventricular hypertrophy (148% of predicted muscle mass). Her arterial blood pressure was normal.

Molecular genetic analysis

This study was approved by the local Ethical Committee at the Cardinal Stephan Wyszynski University in Warsaw (3/2012 CSWUW). DNA isolated from the proband (VI.1) and her healthy parents (IV.3, V.1) was isolated from peripheral blood lymphocytes by the salting-out method, following receipt of the patients' informed consent.

PCR and DNA sequencing

Initially, when we suspected the diagnosis of congenital cataracts, facial dysmorphism and neuropathy (CCFDN), or Marinesco-Sjögren syndrome (MSS), we analysed the *CTDP1* and *SIL1* genes for mutations in the proband and her healthy parents. The 13 coding exons encompassing the intron-exon boundaries of the *CTDP1* gene were amplified using the polymerase chain reaction (PCR) with previously reported primers [19]. The PCR products from the proband and her parents were sequenced directly using a Dynamic terminator cycle sequencing kit (Applied Biosystems) on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). To detect a common CCFDN-causing mutation, i.e. the IVS6+389C>T

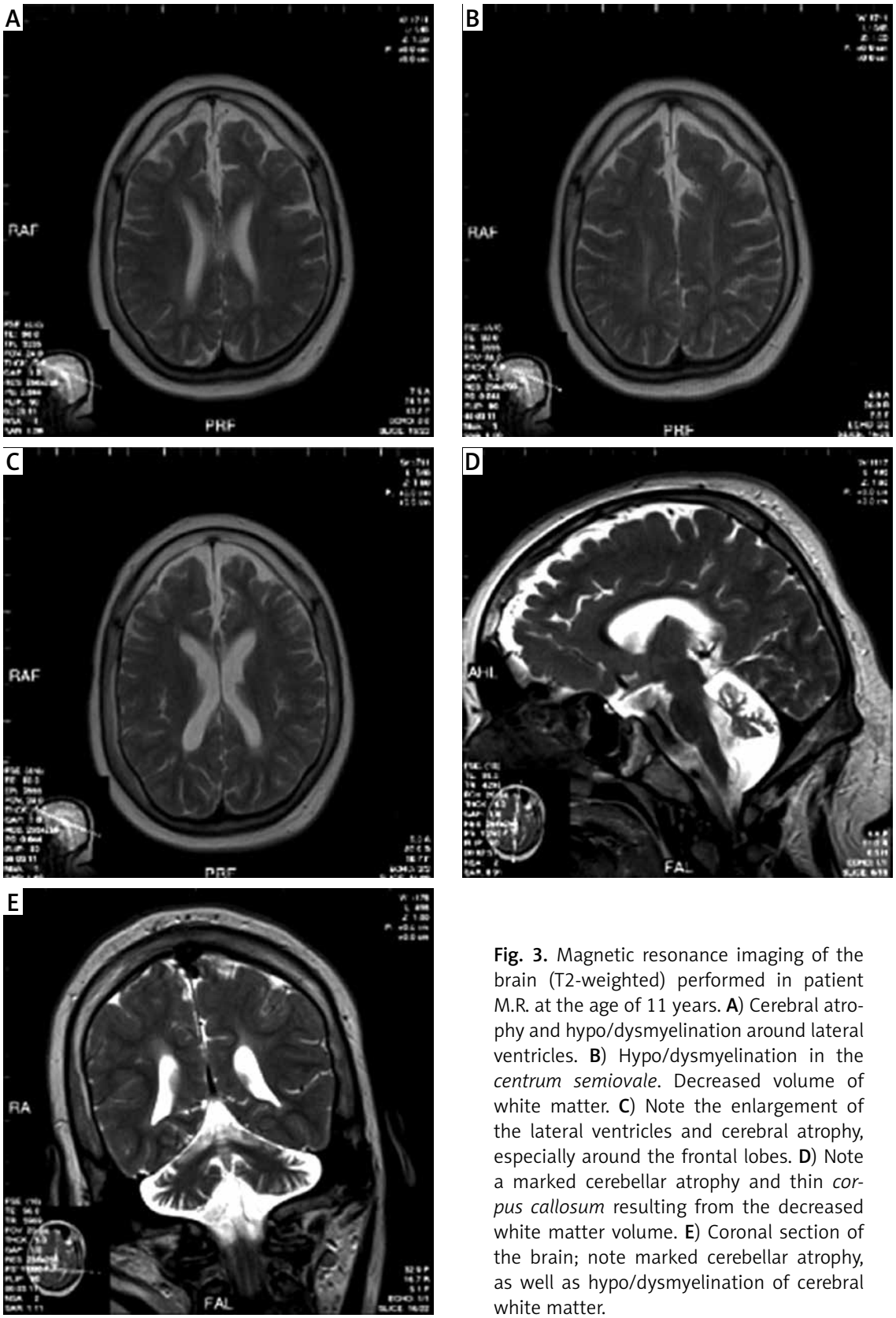


Fig. 3. Magnetic resonance imaging of the brain (T2-weighted) performed in patient M.R. at the age of 11 years. **A**) Cerebral atrophy and hypo/dysmyelination around lateral ventricles. **B**) Hypo/dysmyelination in the *centrum semiovale*. Decreased volume of white matter. **C**) Note the enlargement of the lateral ventricles and cerebral atrophy, especially around the frontal lobes. **D**) Note a marked cerebellar atrophy and thin *corpus callosum* resulting from the decreased white matter volume. **E**) Coronal section of the brain; note marked cerebellar atrophy, as well as hypo/dysmyelination of cerebral white matter.

mutation, a restriction analysis with *Nla* III endonuclease was performed using the forward primer: 5'-CACTGTGTTAGCCAGGATGG-3', and the reverse primer: 5'-GTGCCGTCTGACAGAGATGA-3'. The *SIL1* coding sequence was analysed by direct sequencing of the coding region.

Exome sequencing

Exome sequencing in the proband (VI.1) and parents (IV.3, V.1) was performed in line with the protocol from Illumina's TruSeq Exome Enrichment Guide. The qualified genomic DNA was fragmented by Covaris, ligated with adapters, purified, amplified and hybridized using a Sure Select Human All Exon 50 Mb Kit (Agilent Technologies), following the manufacturer's instructions. For enrichment, hybridized fragments were bound to the streptavidin beads, and non-hybridised fragments were washed out. The enriched library was then loaded on to a HiSeq 2000 instrument (Illumina). Exome sequencing was performed by Intelliseq sp. z o.o., Cracow.

Exome sequence data analysis

The sequence reads were analysed using the Illumina pipeline (adapter sequences were removed and low-quality reads discarded). Reads were processed by Picard, and aligned to a human reference sequence (GRCh37) using Bowtie2. BAM files were obtained for each sample using SAM tools, as well as by removing duplicate reads. SNP calling was then performed using GATK. To identify genes with recessive inheritance (the parents are consanguineous [IV/V]), we filtered data by genotype (the proband as a homozygous alternative and the parents as heterozygous, two other control probes being used as a homozygous reference), before dbSNP annotation was carried out and common variants filtered for. The SIFT tool was used in the functional and conservation prediction of SNPs.

Mutation confirmation by Sanger sequencing

The coding sequence with the intron/exon boundaries of the 7th exon of the *RAB3GAP1* gene was sequenced in family members by means of Sanger sequencing. Primers (Forward: 5'-CAGTTTGGTATTGTAAGGAGAAA-3' and Reverse: 5'-GCTAACAGACTGAACAAACAA-3') were constructed on the basis of the NG_01697.1 genomic and NM_001172435.1 RNA sequences.

Analysis of other genes

To confirm a monogenic basis of the disease in our family we analysed NGS data for all variants in three other genes involved in Warburg micro syndrome diseases, i.e. *RAB3GAP2* (NC_000001.11, NM_012414.3), *RAB18* (NC_000010.11, NM_021252.4) and *TBC1D20* (NC_000020.11, NM_144628.3).

Due to the occurrence of cardiomyopathy in one of the affected sisters, additional analysis of variants in 44 genes associated with cardiomyopathy was performed. Additionally, three genes essential in cardiac homeostasis and mitochondrial fusion (*MFN1* – mitofusin 1, *MFN2* – mitofusin 2 and *OPA1* – optic atrophy 1) were analysed.

We established a list of genes on the basis of articles by J.M. Bos *et al.* 2009, A. Huertas-Vazquea *et al.* 2013, E. Villard *et al.* 2011, K. Stark *et al.* 2010, K.N. Papanicolaou *et al.* 2012 and Y. Chen *et al.* 2011 [4,5,10,15,17,20].

Results

CTDP1 and SIL1 genes

The IVS6+389C>T mutation in the *CTDP1* gene was excluded by means of the RFLP analysis.

The sequencing of the entire *CTDP1* coding sequence (including exon-intron boundaries) revealed two sequence variants. In the proband and her mother, a heterozygous C>T transition at position 1019 resulting (by conceptual translation) in a Thr to Met amino-acid change at codon 340 (T340M) was identified in exon 7 of the *CTDP1* gene. The T340M substitution was not detected in the father of the proband. In the proband and her parents, a heterozygous transition G>A at position 2937 in the non-coding region was identified in exon 13 of the *CTDP1* gene. Analysis of the *SIL1* gene sequence did not reveal any DNA variant which could be considered a pathogenic mutation for Marinesco-Sjögren syndrome (MSS).

Whole-exome sequencing

Whole-exome analysis was performed in patient 1 as well as in her healthy mother and father. DNA from patient 2 was not available for the study. After filtering for common variants (> 1%) present in the dbSNP database, we identified 155 variants of poten-

tial importance. After filtering for nucleotide changes that potentially have a damaging effect on the protein and a high conservation ratio, we obtain only three variants among them, of which only one – c.538 G>T, p.E180X in the *RAB3GAP1* gene – is suitable for the phenotype occurring in patients. Sanger sequencing confirmed the homozygous novel mutation in the proband and a heterozygous one in their healthy parents. The remaining two homozygous variants that were found after filtering were:

– OR13C2 gene (olfactory receptor, family 13, subfamily C, member 2) rs143198170, NC_0009.12: g. 104605112_104605115delGTTA,NM_001004481.1: c. 513_516delTAAC, p. N171Kfs;
– CNOT1 (CCR4-NOT transcription complex, subunit 1): rs5817153, NC_00016.10: g. 58543412delA, NM_016284.4: c. 4434+195delT (isoform a), NM_206999.2 (isoform b): c. 4629delT, p. L1544Cfs, NM_001265612.1: c. 4419+195delT (isoform c).

Analysis of other Warburg syndrome genes

Analysis of other WARBM genes in the proband did not reveal any potentially pathogenic variants. But in the *GAP3RAB2* gene a very rare homozygous variant rs2289189 g.220157863C>G (G allele frequency 0.059), c.3275G>C with heterozygosity of 0.111 was found, this being described in the dbSNP database as a benign polymorphism.

Analysis of the genes involved in cardiomyopathy

The analysis of genes associated with cardiomyopathy revealed many SNPs, from among which only rs365990 (g.23392602A>G; c.3302T>C; p.V1101A) in the *MYH6* gene had been described previously as associated with a higher risk of cardiac conduction and rhythm disorders [4,9]. Moreover, a very rare heterozygous benign polymorphism in the *TTN* gene called rs55842557 (g.178528964G>A with allele A frequency of only 0.008, c.106787C>T, p.T35596I) was found. We identified that variant in the heterozygous state in the proband and her mother, while no mutation was identified in her father. In the *LDB3* gene, a new variant discovered was c.1318 T>C; p.S440P in VI.1, V.1 (heterozygous) and VI.3 (homozygous), but due to the homozygous variant in the healthy father this mutation probably is not associated with cardiomyopathy occurred in VI.1. A few missense benign polymorphisms, as well as numerous silent and intron mutations (not shown) were also found in other analysed genes.

Discussion

Surprisingly, even in the syndromes characterized by a peculiar morphological phenotype, molecular diagnostics may be hampered by the presence of rarely occurring symptoms. The peripheral demyelinating neuropathy and cardiomyopathy are not representative features of WARBM, however peripheral neuropathy was previously reported in two affected sisters harboring mutation within the *TBC1D20* gene [11].

Given the presence of peripheral neuropathy in our patients, we started molecular diagnostics from the syndromes in which peripheral neuropathy is a typical clinical feature.

At the beginning of our study we suspected a diagnosis of congenital cataract facial dysmorphism neuropathy (CCFDN), or Marinesco-Sjögren syndrome (MSS). In a classical phenotype-oriented gene analysis we have excluded the mutations within the *CTDP1* and *SIL1* genes that are respectively causative for CCFDN and MSS.

Due to a lack of conclusive results using the classical Sanger sequencing approach, we decided to perform whole-exome sequencing analysis (WES) in this family.

It is an open question as to whether *RAB3GAP1* mutations alone are sufficient to result in a complete phenotype of WARBM1 patients. To the best of our knowledge, the WES analysis was not widely performed to date in WARBM patients. Thus, the potential impact of other genes has not been analyzed so far. Thus, given the large family of RAB proteins and RAB modulating factors, a question arises in regard to the penetrance of the *RAB3GAP1* mutation. In fact, the patients detected by us manifested with a complete phenotype of WARBM1. In the exome analysis we found only one deleterious *RAB3GAP1* mutation; and no pathogenic mutations have been detected in other RAB and RAB-related genes. Thus, it is highly probable that a single point mutation within the *RAB3GAP1* gene is the one and only cause of WARBM1 disease. This is especially the case given that mutations in the *RAB3GAP1* gene associated with WARBM1 were described in families of very varied ethnic origin, with the number of mutations identified already exceeding 50 [14]. The clinical picture of the WARBM1 in our patients

corresponds with the phenotype described by other authors [7]. However, not typically, in our patients peripheral demyelinating motor-sensory polyneuropathy and cardiomyopathy were identified.

Interestingly, in a series of WARBM individuals with *TBC1D20* mutations, only two sisters were found to have demyelinating polyneuropathy [11]. The occurrence of peripheral demyelinating neuropathy within WARBM-affected patients is hard to estimate, since only in some have electromyographic investigations been reported.

In the whole-exome approach used in this study, we did not find any obvious deleterious mutations in the genes involved in the molecular pathogenesis of hereditary motor and sensory neuropathies (HMSN). Thus, due to a lack of mutations within known HMSN genes, we tend to consider peripheral neuropathy rather as a part of the WARBM1 phenotype than an additional clinical feature associated with a mutation of another gene (overlapping syndrome).

Cerebral and cerebellar atrophy, as well as hypoplasia of the *corpus callosum* seem to be hallmarks of WARBM [3,6,13]. In our patients too, it was possible to observe cerebral and cerebellar atrophy with hypoplasia of the *corpus callosum*. The former sign is a typical finding connected with, and resulting from, hypomyelination. The patients reported by us died in the second decade of life. The nonsense E180X mutation in the *RAB3/GAP1* mutation may be at least partially responsible for the poor clinical outcome in these patients.

In one of our patients, cardiomyopathy with left ventricular hypertrophy was detected. Since early-onset hypertrophic cardiomyopathy is often associated with the mutation of one of the sarcomere genes, we decided to screen the most often mutated genes using an exome approach. We did not detect any pathogenic mutation for the hypertrophic cardiomyopathy. It only proved possible to detect one heterozygous benign polymorphism – rs365990 in the *MYH6* gene – this being previously described as associated with a high risk of cardiac conduction and rhythm disorders [4,8,9]; as well as the heterozygous rare polymorphism rs55842557 in the *TTN* gene. Moreover, polymorphism c.1924-851A>G (rs6730157) in the *RAB3GAP1* gene has been recently described as being associated with effects on total cholesterol and high density lipoprotein cholesterol levels, and with an increased risk of sudden cardiac death [12].

For this reason, it is not definitively possible to preclude hypertrophic cardiomyopathy belonging to the WARBM syndrome phenotype.

Recently, in the consanguineous family with Kurdish-Armenian descent a large deletion encompassing exons 4-15 of the *RAB3GAP1* gene has been found in the siblings with the WARBM1 syndrome. Interestingly, in this pair of patients a mild growth hormone deficiency was detected, and X-ray examination revealed severe osteopenia, which have been not detected to date in WARBM [16]. For molecular diagnostic purposes, the lack or presence of some atypical symptoms should not preclude the molecular analysis of the *RAB* genes. Moreover, due to a limited number of reported patients, the frequency of WARBM-associated symptoms could not be estimated. Noteworthy, clinical variability of WARBM in various age-groups of patients is also unknown. Thus, a minimal set of symptoms which are necessary for WARBM diagnosis and decision concerning molecular diagnostics may be misleading.

In fact, the number of WARBM reported patients is still too low for reliable phenotype-genotype correlations to be derived.

To summarize, the patients reported by us manifest with a severe clinical form of WARBM1 associated with peripheral demyelinating neuropathy and cardiomyopathy.

Due to the unique structure of the family tree in this study (a recessive trait of inheritance and a detected common ancestor in the 6th generation), we were able to prioritize the filtering process in the interpretation of the exome data. The exome analysis identified only three probably important homozygous sequence variants in the proband. Additionally, only one sequence variant was detected in the *RAB-3GAP1* gene whose mutations had been shown previously to segregate with the phenotype of WARBM. Finally, by means of conceptual translation, the identified sequence variant within the *RAB3GAP1* gene resulted in a homozygous nonsense E180X mutation truncating the RAB3GAP1 protein. Our study confirms the usefulness of WES analysis in the pedigrees with an evident recessive trait of inheritance and evidence of the common ancestor in the family.

Because of the non-specificity of signs and symptoms of many disorders, molecular genetics is indispensable in establishing a specific diagnosis. In fact, exome analysis seems to be very serviceable in cases of unknown background. Whole-exome sequenc-

ing in our family gives a chance for the battery of biochemical, imaging and other tests to be omitted. In fact, exome analysis is a cost- and time-effective approach. Most importantly, it also reduces the discomfort of affected patients associated with a long diagnostic process.

Acknowledgements

This study was supported by the Grant for the Polish National Science Centre No 2012/07/B/NZ4/01748 to D.K. We thank Jadwiga Kędzierska for technical support.

Disclosure

Authors report no conflict of interest.

References

1. Ainsworth JR, Morton JE, Good P, Woods CG, George ND, Shield JP, Bradbury J, Henderson MJ, Chhina J. Micro syndrome in Muslim Pakistan children. Ophthalmology 2001; 108: 491-497.
2. Aligianis IA, Johnson CA, Gissen P, Chen D, Hampshire D, Hoffmann K, Maina EN, Morgan NV, Tee L, Morton J, Ainsworth JR, Horn D, Rosser E, Cole TR, Stolte-Dijkstra I, Fieggen K, Clayton-Smith J, Megarbane A, Shield JP, Newbury-Ecob R, Dobyns WB, Graham JM, Jr., Kjaer KW, Warburg M, Bond J, Trembath RC, Harris LW, Takai Y, Mundlos S, Tannahill D, Woods CG, Maher ER. Mutations of the catalytic subunit of RAB3GAP cause Warburg Micro syndrome. Nat Genet 2005; 37: 221-223.
3. Bem D, Yoshimura S, Nunes-Bastos R, Bond FC, Kurian MA, Rahman F, Handley MT, Hadzhiev Y, Masood I, Straatman-Iwanowska AA, Cullinane AR, McNeill A, Pasha SS, Kirby GA, Foster K, Ahmed Z, Morton JE, Williams D, Graham JM, Dobyns WB, Burglen L, Ainsworth JR, Gissen P, Muller F, Maher ER, Barr FA, Aligianis IA. Loss-of-function mutations in RAB18 cause Warburg micro syndrome. Am J Hum Genet 2011; 88: 499-507.
4. Bos JM, Towbin JA, Ackerman MJ. Diagnostic, prognostic, and therapeutic implications of genetic testing for hypertrophic cardiomyopathy. J Am Coll Cardiol 2009; 54: 201-211.
5. Chen Y, Liu Y, Dorn GW. Mitochondrial fusion is essential for organelle function and cardiac homeostasis. Circ Res 2011; 109: 1327-1331.
6. Derbent M, Agras PI, Gedik S, Oto S, Alehan F, Saatci U. Congenital cataract, microphthalmia, hypoplasia of corpus callosum and hypogenitalism: report and review of Micro syndrome. Am J Med Genet A 2004; 128A: 232-234.
7. Handley MT, Morris-Rosendahl DJ, Brown S, Macdonald F, Hardy C, Bem D, Carpanini SM, Borck G, Martorell L, Izzi C, Faravelli F, Accorsi P, Pinelli L, Basel-Vanagaite L, Peretz G, Abdel-Salam GM, Zaki MS, Jansen A, Mowat D, Glass I, Stewart H, Mancini G, Lederer D, Roscioli T, Giuliano F, Plomp AS, Rolfs A, Graham JM, Seemanova E, Poo P, Garcia-Cazorla A, Edery P, Jackson IJ, Maher ER, Aligianis IA. Mutation spectrum in RAB3GAP1, RAB3GAP2, and RAB18 and genotype-phenotype correlations in warburg micro syndrome and Martsolf syndrome. Hum Mutat 2013; 34: 686-696.
8. Holm H, Gudbjartsson DF, Arnar DO, Thorleifsson G, Thorgeirsson G, Stefansdottir H, Gudjonsson SA, Jonasdottir A, Mathiesen EB, Njolstad I, Nyrnes A, Wilsgaard T, Hald EM, Hveem K, Stoltenberg C, Lochen ML, Kong A, Thorsteinsdottir U, Stefansson K. Several common variants modulate heart rate, PR interval and QRS duration. Nat Genet 2010; 42: 117-122.
9. Holm H, Gudbjartsson DF, Sulem P, Masson G, Helgadottir HT, Zanon C, Magnusson OT, Helgason A, Saemundsdottir J, Gylfason A, Stefansdottir H, Gretarsdottir S, Matthiasson SE, Thorgeirsson GM, Jonasdottir A, Sigurdsson A, Stefansson H, Werge T, Rafnar T, Kiemeney LA, Parvez B, Muhammad R, Roden DM, Darbar D, Thorleifsson G, Walters GB, Kong A, Thorsteinsdottir U, Arnar DO, Stefansson K. A rare variant in MYH6 is associated with high risk of sick sinus syndrome. Nat Genet 2011; 43: 316-320.
10. Huertas-Vazquez A, Nelson CP, Guo X, Reinier K, Uy-Evanado A, Teodorescu C, Ayala J, Jerger K, Chugh H, Braund PS, Deloukas P, Hall AS, Balmforth AJ, Jones M, Taylor KD, Pulit SL, Newton-Cheh C, Gunson K, Jui J, Rotter JI, Albert CM, Samani NJ, Chugh SS. Novel loci associated with increased risk of sudden cardiac death in the context of coronary artery disease. PLoS One 2013; 8: e59905.
11. Liegel RP, Handley MT, Ronchetti A, Brown S, Langemeyer L, Linford A, Chang B, Morris-Rosendahl DJ, Carpanini S, Posmyk R, Harthill V, Sheridan E, Abdel-Salam GM, Terhal PA, Faravelli F, Accorsi P, Giordano L, Pinelli L, Hartmann B, Ebert AD, Barr FA, Aligianis IA, Sidjanin DJ. Loss-of-function mutations in TBC1D20 cause cataracts and male infertility in blind sterile mice and Warburg micro syndrome in humans. Am J Hum Genet 2013; 93:1001-1014.
12. Ma L, Yang J, Runesha HB, Tanaka T, Ferrucci L, Bandinelli S, Da Y. Genome-wide association analysis of total cholesterol and high-density lipoprotein cholesterol levels using the Framingham heart study data. BMC Med Genet 2010; 11: 55.
13. Megarbane A, Choueiri R, Bleik J, Mezzina M, Caillaud C. Microcephaly, microphthalmia, congenital cataract, optic atrophy, short stature, hypotonia, severe psychomotor retardation, and cerebral malformations: a second family with micro syndrome or a new syndrome? J Med Genet 1999; 36: 637-640.
14. Morris-Rosendahl DJ, Segel R, Born AP, Conrad C, Loeys B, Brooks SS, Muller L, Zeschnigk C, Botti C, Rabinowitz R, Uyanik G, Crocq MA, Kraus U, Degen I, Faes F. New RAB3GAP1 mutations in patients with Warburg Micro Syndrome from different ethnic backgrounds and a possible founder effect in the Danish. Eur J Hum Genet 2010; 18: 1100-1106.
15. Papanicolaou KN, Kikuchi R, Ngoh GA, Coughlan KA, Dominguez I, Stanley WC, Walsh K. Mitofusins 1 and 2 are essential for postnatal metabolic remodeling in heart. Circ Res 2012; 111: 1012-1026.
16. Picker-Minh S, Busche A, Hartmann B, Spors B, Klopocki E, Hübner C, Horn D, Kaindl AM. Large homozygous *RAB3GAP1* gene microdeletion causes Warburg Micro Syndrome 1. Orphanet J Rare Dis 2014; 9: 113.
17. Stark K, Esslinger UB, Reinhard W, Petrov G, Winkler T, Komajda M, Isnard R, Charron P, Villard E, Cambien F, Tiret L, Aumont MC, Dubourg O, Trochu JN, Fauchier L, DeGroote P,

Richter A, Maisch B, Wichter T, Zollbrecht C, Grassl M, Schunkert H, Linsel-Nitschke P, Erdmann J, Baumert J, Illig T, Klopp N, Wichmann HE, Meisinger C, Koenig W, Lichtner P, Meitinger T, Schillert A, Konig IR, Hetzer R, Heid IM, Regitz-Zagrosek V, Hengstenberg C. Genetic association study identifies HSPB7 as a risk gene for idiopathic dilated cardiomyopathy. PLoS Genet 2010; 6: e1001167.

18. Warburg M, Sjo O, Fledelius HC, Pedersen SA. Autosomal recessive microcephaly, microcornea, congenital cataract, mental retardation, optic atrophy, and hypogenitalism. Micro syndrome. Am J Dis Child 1993; 147: 1309-1312.
19. Varon R, Gooding R, Steglich C, Marns L, Tang H, Angelicheva D, Yong KK, Ambrugger P, Reinhold A, Morar B, Baas F, Kwa M, Tournev I, Guerguelcheva V, Kremensky I, Lochmuller H, Mullner-Eidenbock A, Merlini L, Neumann L, Burger J, Walter M, Swoboda K, Thomas PK, von Moers A, Risch N, Kalaydjieva L. Partial deficiency of the C-terminal-domain phosphatase of RNA polymerase II is associated with congenital cataracts facial dysmorphism neuropathy syndrome. Nat Genet 2003; 35: 185-189.
20. Villard E, Perret C, Gary F, Proust C, Dilanian G, Hengstenberg C, Ruppert V, Arbustini E, Wichter T, Germain M, Dubourg O, Tavazzi L, Aumont MC, DeGroote P, Fauchier L, Trochu JN, Gibelin P, Aupetit JF, Stark K, Erdmann J, Hetzer R, Roberts AM, Barton PJ, Regitz-Zagrosek V, Aslam U, Duboscq-Bidot L, Meyborg M, Maisch B, Madeira H, Waldenstrom A, Galve E, Cleland JG, Dorent R, Roizes G, Zeller T, Blankenberg S, Goodall AH, Cook S, Tregouet DA, Tiret L, Isnard R, Komajda M, Charron P, Cambien F. A genome-wide association study identifies two loci associated with heart failure due to dilated cardiomyopathy. Eur Heart J 2011; 32: 1065-1076.

Folia Neuropathologica

Fahr's syndrome and clinical correlation: a case series and literature review

Michele Pistacchi[1], Manuela Gioulis[2], Flavio Sanson[1], Sandro Zambito Marsala[2]

[1]Neurology Service, Santorso Hospital, Santorso, [2]Department of Neurology, San Martino Hospital, Belluno, Italy

Folia Neuropathol 2016; 54 (3): 282-294 DOI: 10.5114/fn.2016.62538

Abstract

Introduction: *Fahr's disease is characterized by bilateral calcium deposition within the basal ganglia, cerebellar dentate nucleus and subcortical brain white matter. The main clinical manifestations are rigid or hyperkinetic syndrome, mood disorders and cognitive impairment. The correlation between neurological impairment and symmetrical basal ganglia calcification is not so frequent. Aim of the study was to report the results of neurological assessment of three sporadic cases of Fahr's disease highlighting a correlation between the clinical syndrome and neuroimaging.*

Case reports: *Three adults of aged 32, 55 and 70, were studied. They all showed a heterogeneous clinical spectrum. One case developed neuropsychiatric symptoms, whereas the others complained of the tremorigen syndrome. Brain computed tomography scans revealed several calcifications in basal ganglia, cerebellar white matter and dentate nuclei.*

Conclusions: *The pathogenesis of Fahr's disease is probably secondary to the dysfunction of cortico-basal connections and their interhemispheric relations. No significant correlation between calcifications and neurological symptoms is proved.*

Key words: *basal ganglia calcification, hypokinetic syndrome, hypoparathyroidism, hypocalcaemia.*

Introduction

Fahr's disease (FD) is characterized by idiopathic calcification within the basal ganglia, cerebellar dentate nuclei and bilateral white matter, so the term 'bilateral striopallidodentate calcinosis' (BSPDC) appears to be the most appropriate [43,44].

Clinical manifestation occurs at any age without overrepresented ages of onset. Idiopathic BSPDC is clinically heterogeneous. Patients with calcification may exhibit neurological and/or psychiatric symptoms with different degrees of severity and ages of onset. Others can remain asymptomatic throughout life [2]. The prevalence of BSPDC is unknown, but an incidence of basal ganglia calcification ranging from 0.3% to 1.2% has been reported in routine radiological examinations in older reports [27,72] and recently greatly increased (from 1.3% to 20.6%) in recent studies [52,71].

Between 2% and 12% of brain scanners detect the presence of calcification levels within the lymphatic vessels [14-17]. Small "physiological" calcifications, especially located in the globus pallidus, can be evidenced and their prevalence increases with age.

Communicating author

Michele Pistacchi, MD, Neurology Service, Santorso Hospital, 73 Garziere St., 36014 Santorso, Italy, phone: +39 0445 571585, fax: +39 0445 571657, e-mail: michelepistacchi@yahoo.it

When these findings are present in subjects younger than 40 years old, involving simultaneously the globus pallidus, putamen, cerebellar dentate nucleus and white matter (Pale-toothed grooved-calcinosis), they are considered to be pathological.

Normal serum levels of calcium and parathyroid hormone help to differentiate primary familial brain calcification from other disorders, such as hyperthyroidism or hypoparathyroidism.

The disease displays heterogeneity of symptoms, and some individuals with brain calcification can be asymptomatic [19,70]. Primary familial brain calcification is usually inherited in an autosomal dominant manner, and, thus far, mutations in three genes have been found to cause the disease: SLC20A2, PDGFB, and PDGFRB. SLC20A2 encodes for the sodium-dependent phosphate transporter 2 (PiT2). PDGFB and PDGFRB code for the platelet-derived growth factor beta (PDGFb) and its receptor, and the platelet-derived growth factor receptor beta (PDGFR-b), respectively [28,53,69].

The latest study identified in multiple families with PFBC mutations in XPR1, a gene encoding a retroviral receptor with phosphate export function. These mutations are implicating with phosphate homeostasis in PFBC [38].

In published studies, these three genes account for approximately 50% of cases [8,10,23,26,52,54, 73,74]. However, in most of these studies, only the SLC20A2 gene was examined, and only by Sanger sequencing. Comprehensive analyses of all the three genes are scarce as stated by a recent study [67].

One of known causes associated with basal ganglia calcinosis is hypoparathyroidism. Related causes can be infections, such as Epstein-Barr and human immunodeficiency [50], lupus erythematosus [57], perinatal hypoxia [58], radiation or chemotherapy [14], carbon monoxide poisoning and prolonged use of anticonvulsants [15].

The pathophysiological mechanism of calcium and other mineral deposits in extracellular and perivascular zones is still unknown. To date several hypotheses have been formulated, such as interruption of the local blood-brain barrier, altered metabolism within neuronal-glial calcium networks, changes in the extracellular matrix [6]. Calcium and other mineral deposits were found in the walls of capillaries, arterioles, and small veins and in perivascular spaces [23]. Neuronal degeneration and gliosis surrounding these accumulations have been reported [33].

Mucopolysaccharides, traces of aluminium, arsenic, cobalt, copper, molybdenum, iron, lead, manganese, magnesium, phosphorus, silver, and zinc are also present [23,42,51,64].

At the molecular level, calcification generally develops within the vessel wall and in the perivascular space, ultimately extending to the neuron.

Due to defective iron transport and free radical production, tissue damage occurs which leads to the initiation of calcification. It occurs secondarily around a Nidus composed of mucopolysaccharides and related substances. Progressive basal ganglia mineralization tends to compress the vessel lumen, thus initiating a cycle of impaired blood flow, neural tissue injury and mineral deposition. Basal ganglia concretions are recognized as basophilic globules tracking the vessels of arteries, veins and capillaries [59].

Electron microscopy also shows the evidence of a connection between spherical and hemispherical bodies formed in the adventitia of the blood vessel and surrounding glial cells while intima is usually preserved with deposits within the pericytes [30].

Mineral composition of the calcifications varies with the anatomical site and their proximity to vasculature calcifications. It may be due to the abnormal metabolism of calcium and phosphorus while some reports tend to contradict this finding [1,3,29].

In a review of 4219 computed tomography (CT) scans, it was deduced that most calcifications occur bilaterally and symmetrically while a few occur unilaterally and there was no abnormality in metabolism of calcium, denying the pathophysiologic significance of concurrent altered calcium metabolism [31]. Calcifications commonly occur in basal ganglia, thalamus, dentate nucleus, cerebral cortex, cerebellum subcortical white matter, and hippocampus [29,46].

It has been suggested that the hyperintense T2-weighted images in MRI sequences may reflect a slowly progressive metabolic or inflammatory process in the brain with consequent calcification, probably causing the neurologic deficits observed [2]. Single-photon emission computed tomography (SPECT) studies showed a marked blood flow decrease. The criteria for the diagnosis of bilateral striopallidodentate calcinosis (BSPDC) include [37,72] evidence for bilateral basal ganglia calcification; progressive neurological or neuropsychiatric manifestations; onset of symptomatology in the fourth or fifth decade of

life (earlier onset is also likely to occur). There are no biochemical abnormalities and clinical features suggesting the presence of mitochondrial, metabolic disease or other systemic disorders; calcifications are not due to infection, trauma, or toxic causes; moreover, autosomal dominant inheritance (chromosome 14q9) for basal ganglia calcification has been discovered [17] (Table I). Brodaty *et al*. [7] excluded such a locus in the absence of neurological, cognitive and psychiatric symptoms. Further, in hypothyroidism the locus is on 11p [24]; in pseudohypoparathyroidism it is on 20q [37]; in Down's syndrome it is on 21q [48], excluding the possibility that a single gene may be responsible for the calcium and other mineral deposits.

Table I. Pathological conditions associated with calcification of the basal ganglia

Idiopathic hypoparathyroidism
Secondary hypoparathyroidism
Pseudohypoparathyroidism
Pseudo-pseudohypoparathyroidism
Hypothyroidism
Neonatal anoxia
Carbon monoxide poisoning
Lead poisoning
Fahr's disease
Basal ganglia calcification (idiopathic family)
Hastings-James syndrome
Cockayne syndrome
Hyalinosis skin
Tuberous sclerosis
Parkinsonism
Vascular diseases
Cerebral haemorrhage
Radiation therapy
Therapy with methotrexate
Cytomegalic inclusions disease
Encephalitis
Toxoplasmosis
Cysticercosis

Bilateral striopallidodentate calcinosis is frequently suspected in normal aging [2,62]; nevertheless, in the literature there is no clear evidence for establishing when calcifications can be attributed to normal aging or to a pathological process. Considering clinical presentations of BSPDC (Table II) in the presence of family history, diagnosis can be proposed in the absence of one of the first 2 criteria. Whereas when family history is negative, meeting the first 5 criteria is sufficient for the diagnosis of BSPDC only if the calcifications are typical of BSPDC [36,46]. Calcifications are more commonly reported in the globus pallidus; additional reported sites

Table II. Clinical presentations of Fahr's disease as reported in the literature

Radiologic findings
Bilateral symmetrical calcifications of basal ganglia and dentate nucleus
Thalamus, centrum semiovale, cerebellum, and cerebral white matter
Psychiatric symptoms
Cognitive deterioration: dementia, delirium, confusion
Psychotic symptoms: hallucinations, delusions
Catatonia
Irritability
Aggression
Personality disorder and personality changes
Mood disorders: depression, manic symptoms
Anxiety, panic attacks, and obsessive behaviours
Others symptoms
Parkinsonism and movement disorders
Seizures
Headache
Dysarthria
Tremor
Orthostatic hypotension
Vertigo
Paresis
Stroke
Syncope
Ataxia

include putamen, caudate nucleus, internal capsule, dentate nucleus, thalamus, cerebellum, and cerebral white matter [44]. Several cases were diagnosed incidentally [25,61,65,68,72] during routine assessment of psychiatric or somatic symptoms, which may suggest the possibility of underestimated diagnosis of BSPDC.

This study describes three heterogeneous sporadic cases of BSPDC in the absence of demonstrated familiarity. The quantity of calcifications is correlated with the symptomatic status, even though different clinical features are not solely justified by location and severity of calcifications. Thus, the correlation between neurological impairment and symmetrical basal ganglia calcification is not always the same. Brain CT aspects were similar, whereas the clinical neurological manifestations were different. Nowadays, a literature review shows evidence that there is no definite pathogenesis of basal ganglia calcifications.

Genetic tests described in clinical cases were obtained in specialized centres for genetic research on rare diseases.

Clinical cases

Case 1

A 55-year-old man presented with a five-year progressive cognitive decline including dysexecutive syndrome, apathy, along with severe disturbance of reasoning, calculation and sequential tasks.

Moreover, memory loss and depressive mood were expressed without any other psychiatric disorders, such as delusions or hallucinations. Finally, he was unable to perform daily life activities, with decreased verbal fluency, apathy and inability to make decisions. His family history was positive for mood and cognitive impairment; however, brain CT scan did not prove calcifications. Neurological examination disclosed extrapyramidal features with postural tremor and orofacial dyskinesia at lips and tongue.

Mini Mental State Examination (MMSE) score was 23/30 (Italian version); neuropsychological tests showed immediate and recent memory deficits, particularly in remote memory. Despite hospitalisation in psychiatry ward, he continued to wander, interaction was absent, he demonstrated poor self-care and disorganised behaviour (e.g. handling the faeces). Nevertheless, the patient was spatiotemporally oriented and was able to recognise people. He was consistent in his verbal responses with rapid and unclear speech (dysarthric speech) and sometimes words could not be understood. Routine blood tests disclosed normal ionic calcium levels (1.12 mmol/l) including calcium, phosphorus, thyroid hormones and parathormone. Serologic tests for syphilis and HIV were negative. He was treated with carbolithium (600 mg/day) and selective serotonin reuptake inhibitor (SSRI). Brain CT showed extensive bilateral calcifications in the dentate nuclei of the cerebellum, basal ganglia and centrum semiovale (Fig. 1). Brain magnetic resonance imaging confirmed multiple small patchy hypersignals in the same areas (Fig. 2). Molecular diagnosis was made by sequencing of the entire coding region of SLC20A2, PDGFRB, and PDGFB and copy number analysis of SLC20A2 giving normal findings. Bilateral striopallidodentate calcinosis was therefore possible based on clinical features and neuroimaging (bilateral basal ganglia calcification, dysarthria and neuropsychiatric symptoms). The patient showed partial improvement in behavioural symptoms with Carbolithium discontinuation; hence quetiapine (25 mg/day) was added. Symptomatology ameliorated within the 60 following days, specifically with the reduction of orofacial dyskinesia at lips and tongue. Despite partial initial benefits, he showed progression of the disease with a severe depressive syndrome.

Case 2

A 70-year-old woman with a previous history of bronchial asthma and hypertension with neurological symptoms occurring for 20 years, showed mild tremor in the upper limbs. Hypothyroidism was disclosed; T3 and T4 results were always normal. During the following 10-15 years tremor increased progressively up to involving the chin and the voice.

Two years later, memory loss and depressive mood were demonstrated. Mini Mental State Examination (MMSE) score was 23/30 (Italian version); other neuropsychological tests showed deficit in attentional capacity, in spatiotemporal orientation and praxis functions. Complete haematochemical examinations, including thyroid and parathyroid hormones, phosphorus/calcium, liver function tests, complete blood count, along with sedimentation, were normal even when frequently repeated over years. Electroencephalogram (EEG) was unremarkable. Brain CT scan highlighted the symmetrical

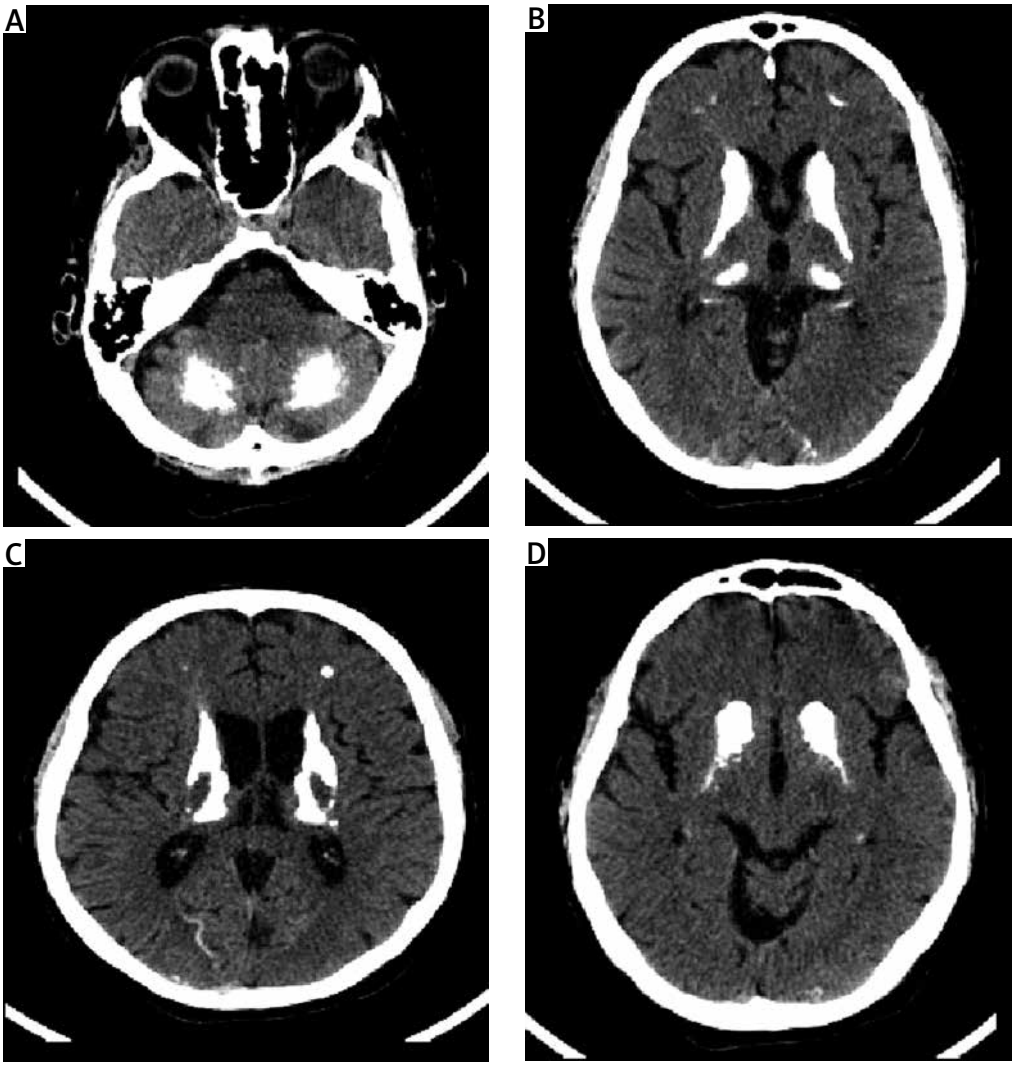


Fig. 1. Computed tomography (CT) findings in patient 1: brain CT shows brain calcification in dentate nuclei of the cerebellum (**A**), basal ganglia, thalamus and cortical (**B-D**).

distribution of mineral deposits in the lenticular nuclei, medial thalamic paraventricular nuclei and in the white matter (Fig. 3). The patient's overall clinical features alongside instrumental examinations allowed to make a diagnosis of BSPDC. After obtaining informed consent, diagnostic genetic testing was performed by sequencing of SLC20A2, PDGFRB, and PDGFB with normal findings. Levodopa was administered but the patient did not tolerate it; beta-blockers could not be used because of bronchial asthma. Within 60 days of alprazolam introduction (3 mg/day), the patient showed partial improvement in behavioural symptoms and essential tremor went into remission.

Regular follow-up during the next three years disclosed tremor recurrence with exclusive involvement of the chin.

Case 3

A 32-year-old man complained of progressive mild tremor at hands in the previous year.

His personal history was positive for Hashimoto thyroiditis. Neurological examination showed only mild rest and action tremor without further signs of extrapyramidal features. Neither dismetry nor any other cerebellar signs were present. General examinations were normal. The patient's relatives were examined and no abnormalities were detected. The thyroid

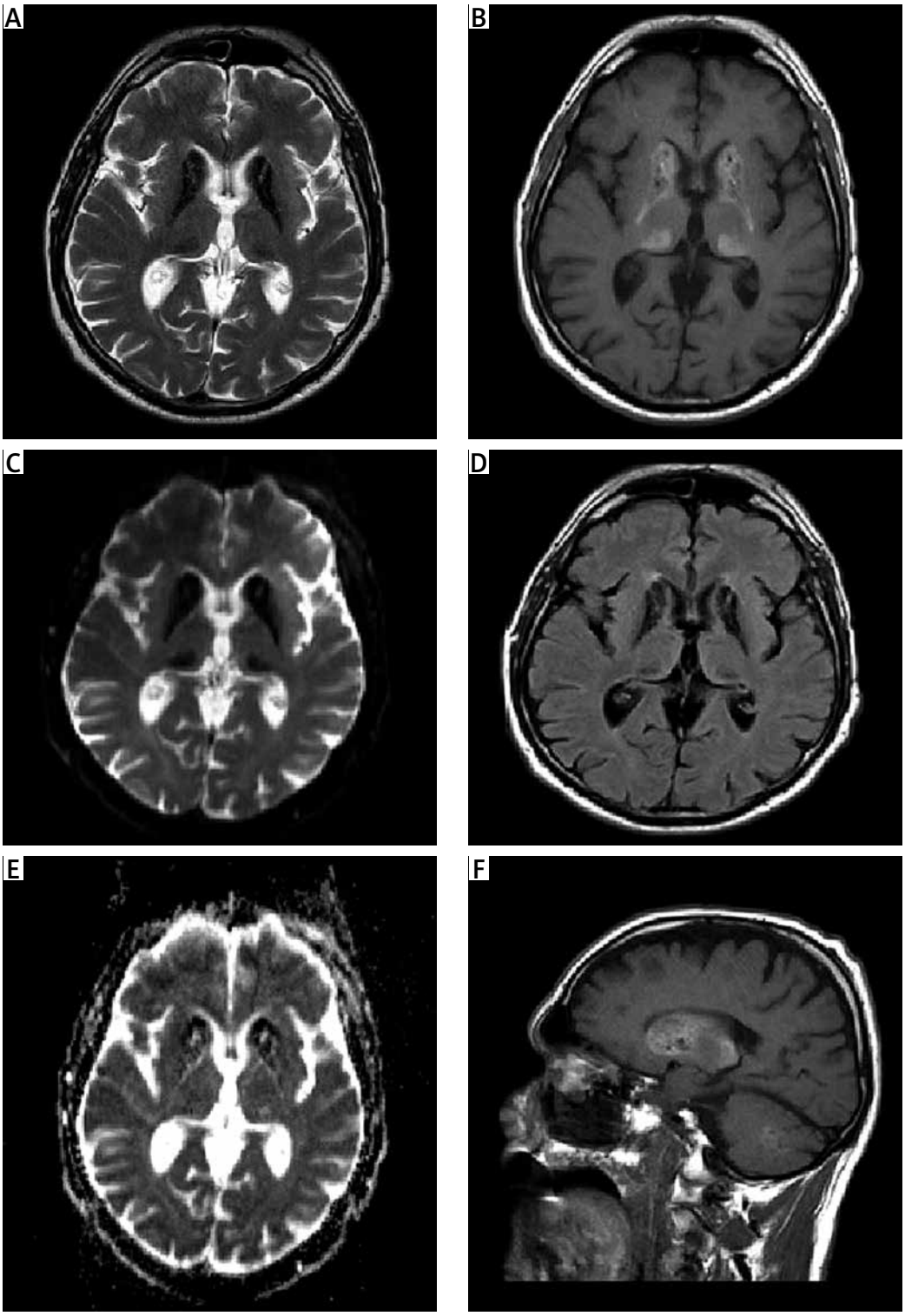


Fig. 2. Magnetic resonance imaging (MRI) findings in patient 1: (**A, D**) T1 and (**B**) T2-weighted image, (**C**) diffusion weighted imaging (DWI) and (**E**) apparent diffusion coefficient (ADC) map, (**F**) fluid-attenuated inversion-recovery (FLAIR) image. Calcified areas show high- or low-intensity signals on MRI T1-weighted images. On T2-weighted image calcification is depicted as low-intensity signals. Multiple small patchy hypersignals on FLAIR sequence. Dark spots are noted on both the DWI and the corresponding ADC map.

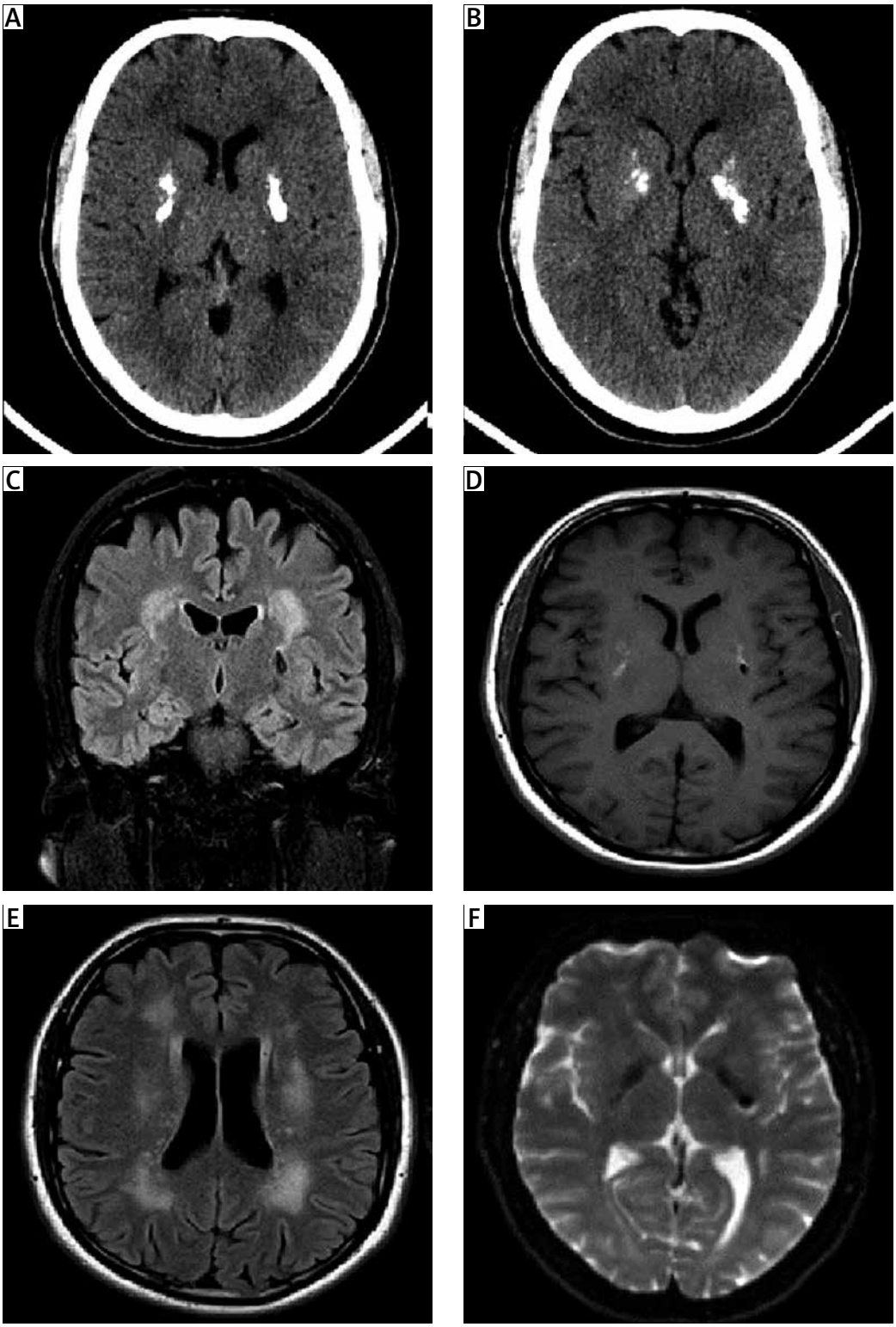


Fig. 3. Computed tomography (CT) and magnetic resonance imaging (MRI) findings in patient 2: brain CT shows a striking high density area in the basal ganglia (**A, B**); MRI calcified areas show hypersignals on FLAIR sequence (**C**), high- or low-intensity signals on MRI T1-weighted images (**D, E**) and dark spots are noted on diffusion weighted imaging (DWI) (**F**).

test, parathyroid hormone, phosphorus/calcium, liver function tests, complete blood count, sedimentation and ionic calcium level were normal. Serologic tests for syphilis and HIV were negative. Cerebrospinal fluid (CSF) analysis was normal. Mini Mental State Examination (MMSE) score was 30/30 (Italian version); other neuropsychological tests were normal. Brain CT scan showed extensive bilateral calcifications in the dentate nuclei of the cerebellum, basal ganglia and bilateral white matter. Magnetic resonance imaging confirmed severe hypointense focal areas with a maximum diameter of about 10 x 7 mm in capsular and peritrigonal areas (Fig. 4). No therapy was administered because of slight symptomatology.

After informed consent to diagnostic genetic testing was obtained, sequencing of SLC20A2, PDGFRB, and PDGFB was performed giving normal findings.

2-year or regular follow-up disclosed no sign of disease progression.

Discussion

Bilateral striopallidodentate calcinosis is mostly associated with a disorder of calcium and phosphate metabolism, especially hypoparathyroidism (HPT) [2,20,55,60]; however, different aetiology must be considered, including infectious, metabolic, and genetic diseases [65].

Our clinical series did not reveal abnormalities in calcium, phosphate, parathyroid levels and other dysmetabolism responsible or calcium deposition.

Furthermore, there was no family history of the disease in all of the three cases reported.

Genetic analysis for SLC20A2, PDGFRB, and PDGFB genes detected no mutations.

Recent reports have highlighted novel hypothesis regarding the possible causes and etiopathological processes of PFBC [21]. In particular, mutations in 3 different genes have been identified as causative agents of PFBC. First, SLC20A2 (OMIM158378) was described in February 2012 as coding for type III sodium-dependent inorganic phosphate (Pi) transporter 2 (PiT-2). The second reported gene, PDGFRB (OMIM173410), was described in January 2013 as encoding 1 of 2 receptors for platelet-derived growth factor. The gene encoding its major ligand, PDGFB (OMIM190040), was the third gene identified in September 2013 [28]. The different mutations in all the 3 genes share loss of function as the probable cause of pathology. Mutations in the SLC20A2 gene lead to accumulation of Pi and subsequently to calcium phosphate deposition. Mutations in PDGFB and PDGFRB also result in calcification through indirect processes. Although the receptor and its ligand are also expressed in neurons, data from selective knockout studies indicate impaired recruitment of pericytes to endothelial cells, resulting in a dysfunctional blood-brain barrier and thereby contributing to brain calcification.

Disease progression is heterogeneous even within the same family. The prevalence of extrapyramidal disorders (parkinsonism, dystonia, dyskinesia) and cerebellar signs (ataxia and dysarthria) is reported; neuropsychiatric symptoms, including schizo-morphous psychosis [9], changes of personality [34], lability of mood and compulsive [18,35,40] obsessive disturbance, were evidenced. Nevertheless a progressive subcortical cognitive impairment could occur.

In a study combining 38 cases recruited through a registry and 61 cases reported in the literature, movement disorders were found as the most common manifestations of BSPDC accounting for 55% of symptomatic patients. The most common movement disorders were parkinsonism (57% of cases), chorea (19%), tremor (8%), dystonia (8%), athetosis (5%) and orofacial dyskinesia (3%).

Measurements of the total volume of calcification suggest a significantly larger amount of calcification in symptomatic patients compared with asymptomatic patients [46].

Neuropsychiatric symptoms can be either the first or the most prominent manifestation ranging from mild concentration or memory impairment, personality and behaviour changes, to psychosis and dementia. About 40% of patients with basal ganglia calcification (BGC) may initially present psychiatric features [5], such as psychotic syndrome and mood changes, but even cognitive disorders are common [13]. Paranoid and psychotic features often begin in patients aged between 20 and 40 [19]. In FD two patterns of psychotic presentation are described: early onset (mean age 30.7 years) with minimal movement disorder and late onset (mean age 49.4 years) associated with dementia and movement disorders [12]. Nevertheless, all the symptoms may vary during the course of disease. Calcification extension and subarachnoid space dilatation correlate with the presence of psychiatric manifestations [41]. Other clinical findings in patients with BSPDC are cranio-cerebral trauma,

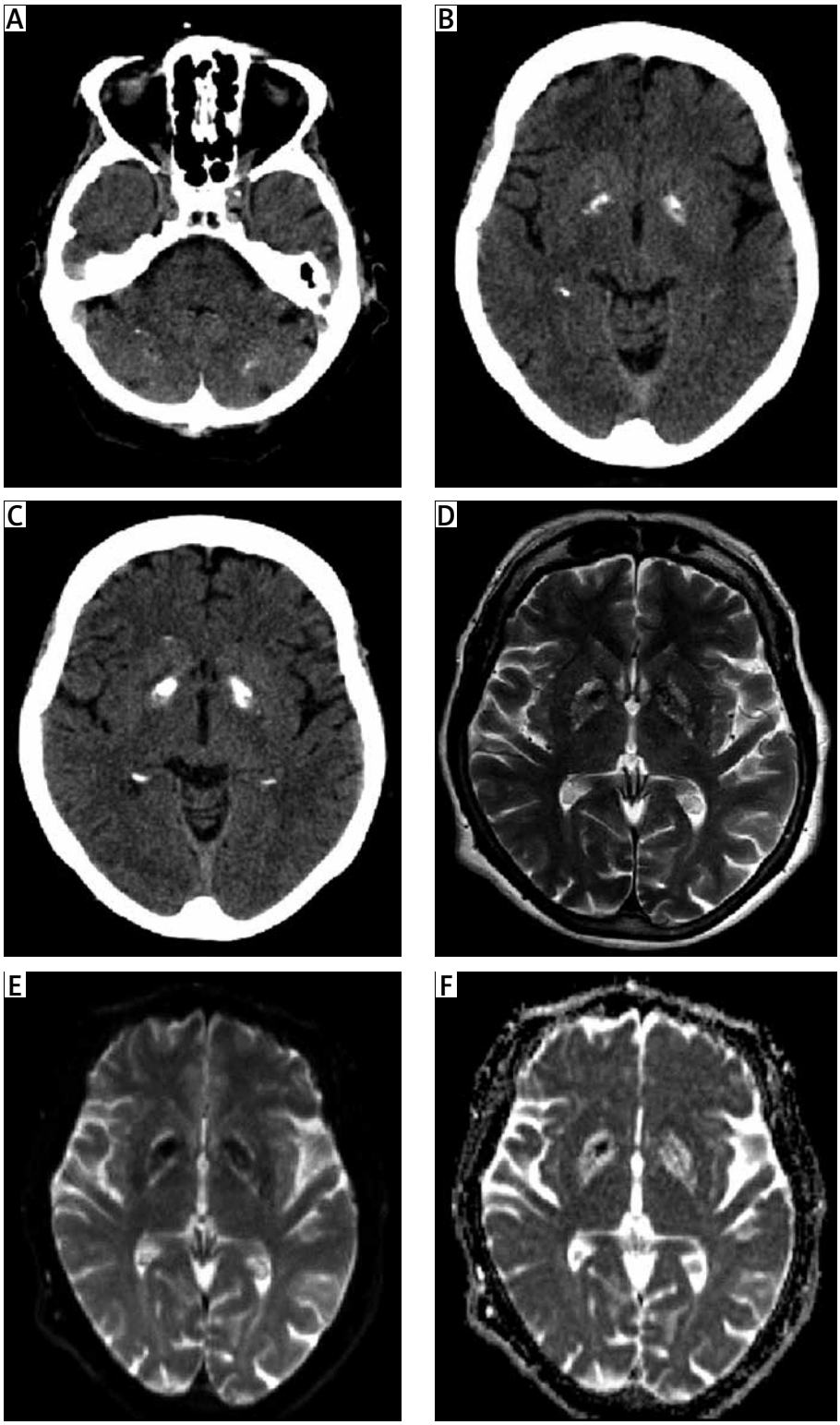


Fig. 4. Computed tomography (CT) and magnetic resonance imaging (MRI) findings in patient 3: an axial view shows a marked high density area in the basal ganglia and dentate nuclei of the cerebellum (**A-C**); MRI calcified areas show hypersignals on FLAIR sequence (**C**), high- or low-intensity signals on MRI T1-weighted images (**D**) and dark spots are noted on diffusion weighted imaging (DWI) (**E**) and the corresponding ADC map (**F**).

stroke, meningitis, encephalitis, brain tumours, cerebral aneurysm, arteriovenous malformation, subdural hematoma, and mastoiditis [36]. Cases presenting with BSPDC and disturbed calcium metabolism were associated with idiopathic hypoparathyroidism, hyperparathyroidism, pseudo-hypoparathyroidism, and postoperative hypoparathyroidism. Patients having parathyroid hormone deficiencies due to thyroidectomy showed more severe mental deterioration [41]. In the three cases reported in our study, the beginning of symptomatology was different and characterised by tremor, psychiatric disorders or movement disorders. The patient with a higher quantity of calcium in TC (case 2) showed onset with predominant psychiatric symptoms, whereas the cases with less (case 2) or earlier (case 3) calcium deposition had prevailing tremor without parkinsonism signs. Besides, aetiology of calcium deposition remains mostly undetermined and no metabolic causes came up in the cases observed.

The exact pathological process responsible for the calcification of brain structures is still poorly understood; it could be secondary to a progressive metabolic or inflammatory process, which subsequently causes the neurological impairments observed [2]. Neuroradiological findings are also different in CT and MRI. The areas involved were basal ganglia, dentate nuclei of cerebellum, medial thalamic paraventricular nuclei, white matter and no correlations were found with clinical manifestations.

These data were confirmed by the literature, especially the non-specificity of lesions for localisation, extension and correlated symptoms.

Moreover, our patients were examined in regular follow-ups and all of them had no progression of symptomatology since the beginning of disease.

It is still unknown why the basal ganglia are the most vulnerable site for calcium deposition, but even in other conditions they are a favourite target, like bilirubin in kernicterus or 1-methyl-4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP) and carbon monoxide causing parkinsonism.

Although correction of dysmetabolic causes, such as hypoparathyroidism or mitochondrial encephalopathy, can improve neuropsychiatric syndrome, there are no specific treatments at the moment that may limit the progression of calcification in the basal ganglia. A report of amelioration using Ca-chelators with antioxidant and Ca-antagonist has still to be confirmed [11]. Currently there is no cure for BSPDC nor is there a standard treatment. The available therapy is only symptomatic and corrections of known aetiology are admitted.

The prognosis is variable and unpredictable. There is no reliable correlation between age, extent of calcium deposits in the brain and neurological deficit. Progressive neurological deterioration generally results in disability and death.

Reduced 25-OH vitamin D_3 with normal levels of $1.25(OH)_2$ vitamin D_3, suggest an inborn error of vitamin D metabolism [47]. Further evaluation of this finding is needed so as to provide a therapeutic solution.

The treatment of BSPDC is directed to the identifiable causes [49]. Especially in HPT, an early treatment can prevent calcification and neurophysiological disorders [20,55,56].

Studies show that psychoses in BSPDC have variable and sometimes null responses to treatment [32].

The treatment targets include symptomatic support. The response to levodopa in those patients with parkinsonian features is reportedly poor. Atypical antipsychotics are preferred for psychiatric symptoms because of the coexistence of the extra pyramidal syndrome in this group of patients.

Treatment of underlying aetiology such as hypoparathyroidism has led to neuropsychiatric improvement, but there are no specific treatments limiting the progression of calcification in the basal ganglia in BSPDC, except a theoretically unconfirmed report of Ca-chelators plus antioxidant and Ca-antagonist benefits [11]. Metal binding proteins and metal-chelating agents (like ammonium tetrathiomolybdate, which is a Cu-chelating agent) have been theoretically suggested as one of the treatment options [22].

Acknowledgements

The authors would like to thank the Santorso Hospital (Santorso, Italy).

Disclosure

Authors report no conflict of interest.

References

1. Asokan AG, D'Souza S, Jeganathan J, Pai S. Fahr's Syndrome – An Interesting Case Presentation. J Clin Diagn Res 2013; 7: 532-533.

2. Avrahami E, Cohn DF, Feibel M, Tadmor R. MRI demonstration and CT correlation of the brain in patients with idiopathic intracerebral calcification. J Neurol 1994; 241: 381-384.
3. Baba Y, Broderick DF, Uitti RJ, Hutton ML, Wszolek ZK. Heredofamilial brain calcinosis syndrome. Mayo Clin Proc 2005; 80: 641-651.
4. Baker M, Strongosky AJ, Sanchez-Contreras MY, Yang S, Ferguson W, Calne DB, Calne S, Stoessl AJ, Allanson JE, Broderick DF, Hutton ML, Dickson DW, Ross OA, Wszolek ZK, Rademarkes R. SLC20A2 and THAP1 deletion in familial basal ganglia calcification with dystonia. Neurogenetics 2013; 15: 23-30.
5. Benke T, Karner E, Seppi K, Delazer M, Marksteiner J, Donnemiller E. Subacute dementia and imaging correlates in a case of Fahr's disease. J Neurol Neurosurg Psychiatry 2004; 75: 1163-1165.
6. Bouras C, Giannakopoulos P, Good PF, Hsu A, Hof PR, Perl DP. A laser microprobe mass analysis of trace elements in brain mineralizations and capillaries in Fahr' s disease. Acta Neuropathol (Berl) 1996; 92: 351-357.
7. Brodaty H, Mitchell P, Luscombe G, Kwok JBJ, Badenhop RF, McKenzie R, Schofield PR. Familial idiopathic basal ganglia calcification (Fahr's disease) without neurological, cognitive and psychiatric symptoms is not linked to the IBGC1 locus on chromosome 14q. Hum Genet 2002; 110: 8-14.
8. Carecchio M, Varrasi C, Barzaghi C, Magistrelli L, Comi C, Garavaglia B, Cantello R. Phenotypic heterogeneity of movement disorders due to intracranial calcifications with or without SLC20A2 mutations. Mov Disord 2014; 29 (Suppl 1): S1-S571.
9. Chabot B, Roulland C, Dollfus S. Schizophrenia and familial idiopathic basal ganglia calcification: a case report. Psychol Med 2001; 31: 741-747.
10. Chen WJ, Yao XP, Zhang QJ, Ni W, He J, Li HF, Liu XY, Zhao GX, Munrong SX, Wang N, Wu ZY. Novel SLC20A2 mutations identified in southern Chinese patients with idiopathic basal ganglia calcification. Gene 2013; 529: 159-162.
11. Cummings JL, Gosenfeld LF, Houlihan JP, McCaffrey T. Neuropsychiatric disturbances associated with idiopathic calcification of the basal ganglia. Biol Psychiatry 1983; 18: 591-601.
12. Cummings JL. Clinical Neuropsychiatry. Grune and Stratton, Orlando 1985; pp. 154-155.
13. Duckett S, Galle P, Escourolle R, Poirier J, Hauw JJ. Presence of zinc, aluminum, magnesium in striopallidodentate (SPD) calcifications (Fahr's disease): electron probe study. Acta Neuropathol (Berl) 1977; 38: 7-10.
14. Fénelon G, Gray F, Paillard F, Thibierge M, Mahieux F, Guillani A. Psychiatry. A prospective study of patients with CT detected pallidal calcifications. J Neurol Neurosurg 1993; 56: 622-625.
15. Fernández-Bouzas A, Ramírez H, Vásquez J, Alonso-Vanegas M, Mendizábal R. Brain calcification and dementia in children treated with radiotherapy and intrathecal metrotexate. J Neurosurg Sci 1992; 36: 2111-2114.
16. Forstl H, Krumm B, Eden S, Kohlmeyer K. Neurological disorders in 166 patients with basal ganglia calcification: a statistical evaluation. J Neurol 1992; 239: 36-38.
17. Geschwind DH, Loginov M, Stern JM. Identification of a locus on chromosome 14q for idiopathic basal ganglia calcification (Fahr disease). Am J Hum Genet 1999; 65: 764-772.
18. Gluck-Vanlaer N, Fallet A, Plas J, Chevalier JF. Depression and calcinosis of the basal ganglia: apropos of a case. Encephale 1996; 22: 127-131.
19. Gomille T, Meyer RA, Falkai P, Gaebel W, Konigshausen T, Christ F. Prevalence and clinical significance of computerized tomography verified idiopathic calcinosis of the basal ganglia. Radiologe 2001; 41: 205-210.
20. Goswami R, Sharma R, Sreenivas V, Gupta N, Ganapathy A, Das S. Prevalence and progression of basal ganglia calcification and its pathogenic mechanism in patients with idiopathic hypoparathyroidism. Clin Endocrinol (Oxf) 2012; 77: 200-206.
21. Hayashi T, Legati A, Nishikawa T, Coppola G. First Japanese family with primary familial brain calcification due to a mutation in the PDGFB gene: an exome analysis study. Clin Neurosci Psychiatry 2015; 69: 77-83.
22. Hozumi I, Kohmura A, Kimura A, Hasegawa T, Honda A, Hayashi Y, Hashimoto K, Yamada M, Sakurai T, Tanaka Y, Satoh M, Inuzuka T. High Levels of Copper, Zinc, Iron and Magnesium, but not Calcium, in the Cerebrospinal Fluid of Patients with Fahr's Disease. Case Rep Neurol 2010; 2: 46-51.
23. Hsu SC, Sears RL, Lemos RR, Quintans B, Huang A, Spiteri E, Nevarez L, Mamah C, Zatz M, Pierce KD, Fullerton JM, Adair JC, Berner JE, Bower M, Brodaty H, Caermona O, Dobricić V, Fogel BL, García-Estevez D, Goldman J, Goudreau JL, Hopfer S, Jancović M, Jaumá S, Jen JC, Kirdlarp S, Klepper J, Kostić V, Lang AE, Linglart A, Maisenbacher MK, Manyam BV, Mazzoni P, Miedzbrodzka Z, Mitarun W, Mitchell PB, Mueller J, Novaković I, Paucar M, Paulson H, Simpson SA, Svenningsson P, Tuite P, Vitek J, Wetchaphanphesat S, Williams C, Yang M, Schofield PR, de Olivera JR, Sobrido MJ, Geschwind DH, Coppola G. Mutations in SLC20A2 are a major cause of familial idiopathic basal ganglia calcification. Neurogenetics 2013; 14: 11-22.
24. Juppner H, Schipani E, Bastepe M, Cole DEC, Lawson ML, Mannstadt M, Hendy GN, Plotkin H, Koshiyama H, Koh T, Crawford JD, Olsen BR, Vikkula M. The gene responsible for pseudohypoparathyroidism type Ib is paternally imprinted and maps in four unrelated kindreds to chromosome 20q13.3. Proc Natl Acad Sci 1998; 95: 11798-11803.
25. Kao YC, Lin MI. Intramuscular hemangioma of the temporalis muscle with incidental finding of bilateral symmetric calcification of the basal ganglia: a case report. Pediatr Neonatol 2010; 51: 296-299.
26. Kasuga K, Konno T, Saito K, Ishihara A, Nishizawa M, Ikeuchi T. A Japanese family with idiopathic basal ganglia calcification with novel SLC20A2 mutation presenting with late-onset hallucination and delusion. J Neurol 2014; 261: 242-244.
27. Kazis AD. Contribution of CT scan to the diagnosis of Fahr's syndrome. Acta Neurol Scand 1985; 71: 206-211.
28. Keller A, Westenberger A, Sobrido MJ, Garcìa-Murias M, Domingo A, Sears RL, Lemos RR, Ordoñez-Ugalde A, Nicolas G, da Cunha JE, Rushing EJ, Hugelshofer M, Wurnig MC, Kaech A, Reimann R, Lohmann K, Dobričić V, Carracedo A, Petrović I, Miyasaki JM, Abakumova I, Mäe MA, Raschperger E, Zatz M, Zschiedrich K, Klepper J, Spiteri E, Prieto JM, Navas I, Preuss M, Dering C, Janković M, Paucar M, Svenningsson P, Salimine-jad K, Khorshid HR, Novaković I, Aguzzi A, Boss A, Le Ber I, Defer G, Hannequin D, Kostić VS, Campion D, Geschwind DH, Cop-

pola G, Betsholtz C, Klein C, Oliveira JR. Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice. Nat Genet 2013; 45: 1077-1082.
29. Kobari M, Nogawa S, Sugimoto Y, Fukuuchi Y. Familial idiopathic brain calcification with autosomal dominant inheritance. Neurology 1997; 48: 645-649.
30. Kobayashi S, Yamadori I, Miki H, Ohmori M. Idiopathic nonarteriosclerotic cerebral calcification (Fahr's disease): an electron microscopic study. Acta Neuropathol (Berl) 1987; 73: 62-66.
31. Koller WC, Cochran JW, Klawans HL. Calcification of the basal ganglia: computerized tomography and clinical correlation. Neurology 1979; 29: 328-333.
32. König P. Psychopathological alterations in cases of symmetrical basal ganglia sclerosis. Biol Psychiatry 1989; 25: 459-468.
33. Kozik M, Kulczycki J. Laser-spectrographic analysis of the cation content in Fahr's syndrome. Arch Psychiatr Nervenkr (1970) 1978; 225: 135-142.
34. Lauterbach EC, Cummings JL, Duffy J, Coffey CE, Kaufer D, Lovell M, Malloy P, Reeve A, Royall DR, Rummans TA, Salloway SP. Neuropsychiatric correlates and treatment of lenticulostriatal diseases: a review of the literature and overview of research opportunities in Huntington's, Wilson's and Fahr's diseases. A report of the ANPA Committee on Research. American Neuropsychiatric Association. J Neuropsychiatry Clin Neurosci 1998; 10: 249-266.
35. Lawrence AD, Watkins LHA, Sahakian BJ, Hodges JR, Robbins TW. Visual object and visuospatial cognition in Huntington s disease: implications for information processing in corticostriatal circuits. Brain 2000; 123: 1349-1364.
36. Lazar M, Ion DA, Streinu-Cercel A, Badarau AI. Fahr's syndrome: diagnosis issues in patients with unknown family history of disease. Rom J Morphol Embryol 2009; 50: 425-428.
37. Lebo RV, Cheung M-C, Bruce BD, Riccardi VM, Kao F-T, Kan YW. Mapping parathyroid hormone, beta-globin, insulin, and LDH-A genes within the human chromosome 11 short arm by spot blotting sorted chromosomes. Hum Genet 1985; 69: 316-320.
38. Legati A, Giovannini D, Nicolas G, López-Sánchez U, Quintáns B, Oliveira JR, Sears RL, Ramos EM, Spiteri E, Sobrido MJ, Carracedo Á, Castro-Fernández C, Cubizolle S, Fogel BL, Goizet C, Jen JC, Kirdlarp S, Lang AE, Miedzybrodzka Z, Mitarnun W, Paucar M, Paulson H, Pariente J, Richard AC, Salins NS, Simpson SA, Striano P, Svenningsson P, Tison F, Unni VK, Vanakker O, Wessels MW, Wetchaphanphesat S, Yang M, Boller F, Campion D, Hannequin D, Sitbon M, Geschwind DH, Battini JL, Coppola G. Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. Nat Genet 2015; 47: 579-581.
39. Lemos R, Oliveira M, Oliveira J. Reporting a new mutation at the SLC20A2 gene in familial idiopathic basal ganglia calcification. Eur J Neurol 2013; 20: e43-44.
40. Lombardi WJ, Gross RE, Trepanier LL, Lang AE, Lozano AM, Saint-Cyr JA. Relationship of lesion location to cognitive outcome following microelectrode-guided pallidotomy for Parkinson's disease. Support for the existence of cognitive circuits in the human pallidum. Brain 2000; 123: 746-758.
41. López-Villegas D, Kulisevsky J, Deus J, Junque C, Pujol J, Guardia E, Grau JM. Neuropsychological alterationsin patients with computed tomography detected basal ganglia calcification. Arch Neurol 1996; 53: 251-256.
42. Lowenthal A, Bruyn GW. Calcification of the striopallidodentate system. In: Handbook of Clinical Neurology. Vol. 6. Diseases of the Basal Ganglia. Vinkin PJ, Bruyn GW (eds.). North-Holland, Amsterdam 1968; p.703-725; Chapter 27.
43. Manyam BV. Bilateral striopallidodentate calcinosis: a proposed classification of genetic and secondary causes. Mov Disord 1990; 5 (Suppl. 1): 94.
44. Manyam BV, Bhatt MH, Moore WD, Develschoward AB, Anderson DR, Caline DB. Bilateral striopallidodentate calcinosis: cerebrospinal fluid, imaging, and electrophysiological studies. Ann Neurol 1992; 31: 379-384.
45. Manyam BV. What is and what is not 'Fahr's disease'. Parkinsonism Relat Disord 2005; 11: 73-80.
46. Malik R, Pandya V, Naik D. Fahr disease – a rare neurodegenerative disorder. Indian J Radiol Imaging 2004; 14: 383-384.
47. Martinelli P, Giuliani S, Ippoliti M, Martinelli A, Sforza A, Ferrari S. Familial idiopathic strio-pallido-dentate calcifications with late onset extrapyramidal syndrome. Mov Disord 1993; 8: 220-222.
48. McCormick M, Schinzel A, Petersen M, Stetten G, Driscoll D, Cantu E, Tranebjaerg L, Mikkelsen M, Watkins P, Antonarakis S. Molecular genetic approach to the characterization of the Down syndrome region of chromosome 21. Genomics 1989; 5: 325-331.
49. Mitchell DM, Regan S, Cooley MR, Lauter KB, Vrla MC, Becker CB, Burnett-Bowie SA, Mannstadt M. Long-term follow-up of patients with hypoparathyroidism. J Clin Endocrinol Metab 2012; 97: 4507-4514.
50. Morita M, Tsuge I, Matsuoka H, Ito J, Iotsu T, Yamamoto M, Morishima T. Calcification in the basal ganglia with chronic active Epstein-Barr virus infection. Neurology 1998; 50: 1485-1488.
51. Neumann MA. Iron and calcium dysmetabolism in the brain with special predilection for globus pallidus and cerebellum. J Neuropath Exp Neurol 1963; 22: 148-168.
52. Nicolas G, Pottier C, Charbonnier C, Guyant-Maréchal L, Le Ber I, Pariente J, Labauge P, Ayrignac X, Defebvre L, Maltête D, Martinaud O, Lefaucheur R, Guillin O, Wallon D, Chaumette B, Rondepierre P, Derache N, Fromager G, Schaeffer S, Krystkowiak P, Verny C, Jurici S, Sauvée M, Vérin M, Lebouvier T, Rouaud O, Thauvin-Robinet C, Rousseau S, Rovelet-Lecrux A, Frebourg T, Campion D, Hannequin D. French IBGC Study Group Phenotypic spectrum of probable and genetically-confirmed idiopathic basal ganglia calcification. Brain 2013; 136 (Pt 11): 3395-3407.
53. Nicolas G, Pottier C, Maltete D, Coutant S, Rovelet-Lecrux A, Legallic S, Rousseau S, Vaschalde Y, Guyant-Maréchal L, Augustin J, Martinaud O, Defebvre L, Krystkowiak P, Pariente J, Clanet M, Labauge P, Ayrignac X, Lefaucheur R, Le Ber I, Frébourg T, Hannequin D, Campion D. Mutation of the PDGFRB gene as a cause of idiopathic basal ganglia calcification. Neurology 2013; 80: 181-187.
54. Nicolas G, Rovelet-Lecrux A, Pottier C, Martinaud O, Wallon D, Vernier L, Landemore G, Chapon F, Prieto-Morin C, Tournier-Lasserve E, Frébourg T, Campion D, Hannequin D. PDGFB partial deletion: a new, rare mechanism causing brain calcifi-

cation with leukoencephalopathy. J Mol Neurosci 2014; 53: 171-175.
55. Nicolau Ramis J, Espino Ibáñez A, Rivera Irigoín R, Artigas CF, Masmiquel Comas L. Extrapyramidal symptoms due to calcinosis cerebri in a patient with unknown primary hypoparathyroidism. Endocrinol Nutr 2012; 59: 69-71.
56. Rastogi R, Singh AK, Rastogi UC, Mohan C, Rastogi V. Fahr's syndrome: a rare clinico-radiologic entity. Med J Armed Forces India 2011; 67: 159-161.
57. Raymond AA, Zariah AA, Samad SA, Chin CN, Kong NC. Brain calcification in patients with cerebral lupus. Lupus 1996; 5: 123-128.
58. Rodríguez MJ, Ursu G, Bernal F, Cusì V, Mahy N. Perinatal human hypoxia-ischemia vulnerability correlates with brain calcification. Neurobiol Dis 2001; 8: 59-68.
59. Saleem S, Aslam HM, Anwar M, Anwar S, Saleem M, Saleem A, Rehmani MA. Fahr's syndrome: literature review of current evidence. Orphanet J Rare Dis 2013; 8: 156.
60. Senoglu M, Tuncel D, Orhan FO, Yuksel Z, Gokçe M. Fahr's syndrome: a report of two cases. Firat Tip Dergisi 2007; 12: 70-72.
61. Shakibai SV, Johnson JP, Bourgeois JA. Paranoid delusions and cognitive impairment suggesting Fahr's disease. Psychosomatics 2005; 46: 569-572.
62. Simoni M, Pantoni L, Pracucci G, Palmetz B, Guo X, Gustafson D, Skoog J. Prevalence of CT-detected cerebral abnormalities in an elderly Swedish population sample. Acta Neurol Scand 2008; 118: 260-267.
63. Skvortsov IA, Rudenskaia GE, Karaseva AN, Vel'tishchev IuE. Effectiveness of therapeutic use of complexones in various diseases of the extrapyramidal system in children. Zh Nevropatol Psikhiatr Im S S Korsakova 1987; 87: 1457-1462.
64. Smeyers-Verbeke J, Michotte Y, Pelsmaeckers J, Lowenthal A, Massart DL, Dekegel D, Karcher D. The chemical composition of idiopathic nonarteriosclerotic cerebral calcifications. Neurology 1975; 25: 48-57.
65. Srivastava S, Bhatia MS, Sharma V, Mahajan S, Rajender G. Fahr's Disease: An Incidental Finding in a Case Presenting with Psychosis. German Journal of Psychiatry 2010; 13: 86-90.
66. Swami A, Kar G. Intracranial hemorrhage revealing pseudohypoparathyroidism as a cause of Fahr syndrome. Case Rep Neurol Med 2011; 2011: 407567.
67. Taglia I, Mignarri A, Olgiati S, Menci E, Petrocelli PL, Breedveld GJ, Scaglione C, Martinelli P, Federico A, Bonifati V, Dotti MT. Primary Familial Brain Calcification: Genetic Analysis and Clinical Spectrum. Mov Disord 2014; 29: 1691-1695.
68. Trautner RJ, Cummings JL, Read SL, Benson DF. Idiopathic basal ganglia calcification and organic mood disorder. Am J Psychiatry 1988; 145: 350-353.
69. Wang C, Li Y, Shi L, Ren J, Patti M, Wang T, de Oliveira JR, Sobrido MJ, Quintáns B, Baquero M, Cui X, Zhang XY, Wang L, Xu H, Wang J, Yao J, Dai X, Liu J, Zhang L, Ma H, Gao Y, Ma X, Feng S, Liu M, Wang QK, Forster IC, Zhang X, Liu JY. Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. Nat Genet 2012; 44: 254-256.
70. Wider C, Dickson DW, Schweitzer KJ, Broderick DK, Wszolek ZK. Familial idiopathic basal ganglia calcification: a challenging clinical-pathological correlation. J Neurol 2009; 256: 839-842.
71. Yamada M, Asano T, Okamoto K, Hayashi Y, Kanematsu M, Hoshi H, Akaiwa Y, Shimohata T, Nishizawa M, Inuzuka T, Hozumi I. High frequency of calcification in basal ganglia on brain computed tomography images in Japanese older adults. Geriatr Gerontol Int 2013; 13: 706-710.
72. Yamada N, Hayashi T. Asymptomatic familial basal ganglia calcification with autosomal dominant inheritance: a family report. No To Hattatsu 2000; 32: 515-519.
73. Yamada M, Masaki T, Mari T, Kobayashi S, Taguchi J, Takashima S, Tanaka K, Touge T, Hatsuta H, Murayama S, Hayashi Y, Kaneko M, Ishiura H, Mitsui J, Atsuta N, Sobue G, Shimozawa N, Inuzuka T, Tsuji S, Hozumi I. Evaluation of SLC20A2 mutations that cause idiopathic basal ganglia calcification in Japan. Neurology 2014; 82: 705-712.
74. Zhang Y, Guo X, Wu A. Association between a novel mutation in SLC20A2 and familial idiopathic basal ganglia calcification. PLoS One 2013; 8: e57060.

Folia Neuropathologica

Combination of acid phosphatase positivity and rimmed vacuoles as useful markers in the diagnosis of adult-onset Pompe disease lacking specific clinical and pathological features

Claire Dolfus[1], Jean-Philippe Simon[2], Gérard Landemore[1], François Leroy[3], Françoise Chapon[2]

[1]Department of Pathology, University Hospital of Caen, [2]Department of Pathology and Neuromuscular Competence Center, University Hospital of Caen, [3]Neuromuscular Competence Center, University Hospital of Caen, France

Folia Neuropathol 2016; 54 (3): 295-302 DOI: 10.5114/fn.2016.62539

Abstract

Introduction: *The clinical and histological presentations of the adult form of Pompe disease may be atypical. In such cases, identifying histological signs that point to the diagnosis would be crucial to avoid a delay in care. The aim of our study was to investigate the presence of rimmed vacuoles and acid phosphatase positivity in muscle biopsies of patients with late-onset Pompe disease.*

Material and methods: *We retrospectively studied muscle biopsies of all cases of the adult form of Pompe disease diagnosed at the University Hospital of Caen. Three of these four cases showed atypical clinical signs, and diagnosis was established tardily based on family history or systematic analysis of acid maltase activity.*

Results: *All biopsies showed some rimmed vacuoles. The acid phosphatase reaction showed positive inclusions and labelled vacuoles in biopsies of all patients.*

Conclusions: *The presence of rimmed vacuoles and acid phosphatase positivity in muscle biopsy should suggest the diagnosis of the adult form of Pompe disease, this is decisive since effective therapy is available.*

Key words: *muscle, storage, alpha-glucosidase, Pompe, lysosomal, pathology, rimmed, vacuole, phosphatase.*

Introduction

Pompe disease is an autosomal recessive glycogen (glycogen storage disease type II) that results from a deficiency of alpha-glucosidase (GAA) or acid maltase, a lysosomal enzyme. This enzymatic deficiency generates an abnormal glycogen accumulation mainly in muscle cells. Different phenotypes are reported: infantile, juvenile and adult [3].

The adult form is classically characterized by slowly progressive motor weakness that predominantly affects the pelvic limb girdle and by respiratory failure with diaphragmatic paralysis [5]. Since the disease progression can be slowed down by a replacement enzyme therapy (Myozyme™) [14], it is important to get an early diagnosis. The clinical diagnosis is confirmed by an assay of GAA enzyme activity in blood [1]. In some cases, however, the clinical presentation is not obvious, specific or misleading so examination of muscle biopsy is required to raise the suspicion of Pompe disease [13].

Communicating author

Françoise Chapon, Department of Pathology and Neuromuscular Competence Center, University Hospital of Caen, Rochambelles St., 13032 CAEN, France, phone: 33675211653, fax: 33231065063, e-mail: chapon-f@chu-caen.fr

Histological aspects of Pompe disease in striated muscle are usually cytoplasmic vacuoles, periodic acid Schiff (PAS) positive and highly reactive to acid phosphatase indicating their lysosomal nature [17]. The ultrastructural study confirms the glycogenic nature of the vacuolar content [8]. Other lesions such as segmented fibers, fibers in necrosis/regeneration [15] or acid phosphatase positive inclusions are more rarely observed [18]. An inflammatory lymphocytic infiltrate or a type grouping can mimic chronic myositis or neurogenic damage [6,15]. Finally, the muscle biopsy can be normal [13].

Surprisingly, we observed, on a late-onset Pompe disease muscle biopsy, rimmed vacuoles in the cytoplasm of muscle fibers. This histologic appearance is not specific and is conventionally a diagnostic criterion of sporadic (s-IBM) or hereditary (h-IBM) inclusion body myositis, oculopharyngeal muscular dystrophy and few myofibrillar myopathies and distal myopathies [2,9].

The aim of our work has been to evaluate the rimmed vacuoles and acid-phosphatase positivity diagnostic interests as new diagnostic histological markers in late-onset Pompe disease. These histological elements have been sought on muscle biopsies from our patients affected by this insidious disease.

Material and methods

Patients

Patient 1 was diagnosed at the age of 34 years following a respiratory distress episode, initially attributed to pulmonary emphysema with large bubbles for which he was operated. Pulmonary function tests, however, put in evidence a restrictive respiratory secondary to a lordosis, a scoliosis and a diaphragmatic paralysis. In addition, clinical examination revealed severe motor deficit in the limb girdle muscles that has evolved over the last twenty years, and an axial deficit. The 10-meter walking test was achieved in 9 seconds, and the Brooke and Vignos scale was scored at 2. Creatine phosphokinase (CPK) was increased to 2 times normal. The electromyography (EMG) showed myogenic plots. The activity of acid maltase was very low and genetic analysis showed two different mutations in the *GAA* gene (IVS1-13T>G and c.2182_2183delT). The patient was treated by enzyme replacement therapy Myozyme™ for 4 years which improved and stabilized his muscular capacity, including breathing.

Patient 2, seen for consultation at the age of 62, showed a typical picture of late-onset Pompe disease. He presented at the age of 38 years for spinal pain secondary to an axial deficit responsible for a hyperlordosis associated with limb girdle muscle weakness, and a face deficit that gradually worsened. At the age of 59, a respiratory failure, secondary to diaphragmatic paralysis, required respiratory support. Creatine phosphokinase was increased to 2 times normal. Functional respiratory tests confirmed diaphragmatic paralysis. The biopsy showed typical lesions of Pompe disease such as numerous vacuoles PAS positive. Unexpectedly, rimmed vacuoles were also found. Biochemical analyses indicated a severe acid maltase deficiency (13%), and genetic analysis revealed two compound heterozygous pathogenic mutations in the *GAA* gene (IVS1-13T>G; c.1447G>T). The patient died at the age of 65 before the initiation of treatment with Myozyme™.

Patient 3 is a sister of patient 2, 14 years younger. She was seen for the first time at the age of 45 for a bilateral ptosis associated with proximal muscle weakness of the lower limbs, which started around the age of 40. Further assessment revealed an increase in CPK to 2 times normal, and normal electromyography; inconspicuous inequality of fibers size with some segmentations and rimmed vacuoles were found on muscle biopsy. In view of these findings, a diagnosis of oculopharyngeal muscular dystrophy was made. Unfortunately, *PABPN1* gene screening did not find any triplet expansion. She then presented an acromegaly secondary to a pituitary microadenoma, as her mother, and a type 2 diabetes. The evolution was marked by the appearance of ophthalmoplegia, worsening of the limb girdle deficit, and a respiratory failure secondary to a diaphragmatic paralysis. The diagnosis of Pompe disease was then made following that of her brother made a year earlier. Acid maltase activity assessed then was very low (12%), and genetic analysis showed heterozygote mutations on the *GAA* gene (IVS1-13T>G; c.1447G>T) identical to those observed in her brother. A pathogenic mutation in the *LMNA* gene (c.1930C>T) was also found. She died at the age of 56 after 42 administrations of Myozyme™ over 21 months.

Patient 4, diabetic and hypertensive, was diagnosed at the age of 60 when she was seen for the first time for walking and stair climbing difficulties that began at the age of 38 years and evolved very

slowly. Physical examination revealed mild proximal weakness of all four limbs and severe axial deficit. Creatine phosphokinase was high, at 4 times normal. On the two muscle biopsies that were performed, only rare rimmed vacuoles were found. The patient did not show any respiratory signs, and respiratory function tests were normal. Acid maltase deficiency was discovered following her inclusion in a research protocol in which patients with an atypical clinical picture were all tested for putative Pompe disease [7]. Diagnosis of Pompe disease was then confirmed by the presence of two mutations in the *GAA* gene (c.1-45T>G in intron 1 and c.1655T>C in exon 12). Once the patient has begun the treatment with Myozyme™, a dramatic improvement occurred over the next 18 months, followed by stabilization of the disease.

Frozen sections

All muscle biopsies were frozen and stored at –80°C. Cuts of 8 to 12 microns were formed in the cryostat. The stains and enzymatic reactions carried out were: haematoxylin-eosin, Gomori trichrome, periodic acid-Schiff (PAS), acid phosphatase, ATPase, and oxidative reactions.

Electron microscopy

The samples were successively fixed by glutaraldehyde, post-fixed with osmium tetroxide, dehydrated, and embedded in resin. Semi-thin and ultrafine sections were then made.

Semi-thin sections were stained with toluidine blue, and also with PAS in patient 2.

Table I. Muscle biopsies of patients affected by the adult form of Pompe disease – histology and electronic microscopy

	Patient 1 Left quadriceps	Patient 2 Left quadriceps	Patient 3 Left deltoid	Patient 4 Right deltoid	Patient 4 Left quadriceps
Histology					
Inequality of fiber size	++	++	+	+	++
Necrosis	–	+	–	–	–
Inflammatory infiltrate	–	–	–	–	–
Internalized nuclei	+	++	+	–	–
Segmentation	+	+	–	–	Rare
Common vacuoles	+	+++	+	+	+
Rimmed vacuoles	+	++	+	+	+
Eosinophilic inclusions	–	+	+	–	–
PAS on frozen block	–	+ On a limited sector	–	–	–
PAS on resin block		Positivity of all vacuoles			
Predominance of one type of fiber	Type I	–	–	–	–
Acid phosphatase reaction	Positive vacuoles and inclusions	Positive vacuoles and inclusions	Positive vacuoles and inclusions	Positive vacuoles	Positive vacuoles
Electronic microscopy					
Free cytoplasmic glycogene	+++	++	+	++	++
Autophagic vacuoles	+	+++	+	++	++
Intralysosomial glycogene accumulation	+	++	+	+	++
Anormal mitochondria	–	+	–	–	–
Under sarcolemmal accumulation of mitochondria	+	++	+	+	++
Myofibrilla alteration	+	++	+	+	++

Results

Histological findings on muscle biopsies are shown in Table I. Particularly relevant is the lack of positive PAS vacuoles for 3 of the 4 patients, and the presence of numerous vacuoles in patient 2 in favour of glycogen excess (Fig. 1A), PAS positivity was observed only on semi-thin sections of this patient. Biopsies of the other patients showed some vacuoles, but they were PAS negative.

Furthermore, rimmed vacuoles were present on all biopsies, obviously predominant in patient 2's biopsy (Table I and Fig. 1B-D). The acid phosphatase reaction showed positive inclusions and labelled vacuoles in biopsies of all patients (Fig. 2A-C).

In electronic microscopy, all biopsies included free cytoplasmic glycogen, glycogen loaded lysosomes and autophagic vacuoles (Fig. 3A-B). The lesions were more marked on patient 2's biopsy. Biopsy of case 1 was characterized by a predominance of glycogen in free form.

Discussion

Our study shows acid phosphatase positive inclusions and rimmed vacuoles in the biopsy of all four patients.

Acid phosphatase positive inclusions or reduced body-like inclusions, that appear as dense globules to electrons in electronic microscopy, are described in the adult form of Pompe disease [16,18]. According to Feeney *et al*., these structures correspond to lipofuscin inclusions [4]. The present study confirms that the acid phosphatase positive inclusions can be a useful diagnostic marker for Pompe disease in cases without histological evidence, as previously suggested by Tsuburaya *et al*. [18]. Rimmed vacuoles were present in biopsies of all our patients with late-onset Pompe disease. The rimmed vacuoles are small vacuoles highlighted by basophil grains on hematoxylin-eosin and red grains on Gomori trichrome [12]. According to Nishino *et al*. [10,12],

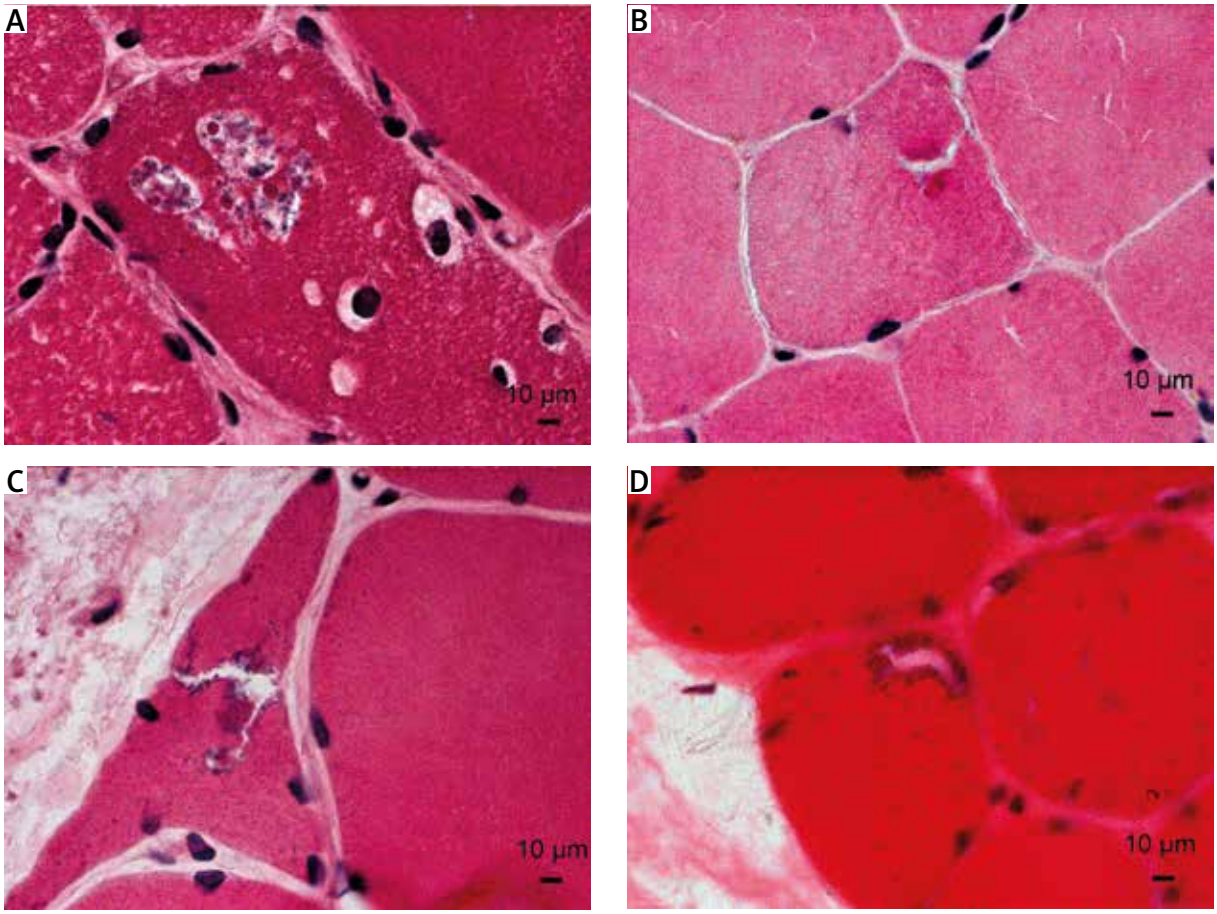


Fig. 1. Hematoxylin-eosin: **A**) Common vacuoles and vacuoles with a basophilic material on hematoxylin and eosin (patient 2), **B-D**) Rimmed vacuoles (patient 4, 1 and 3).

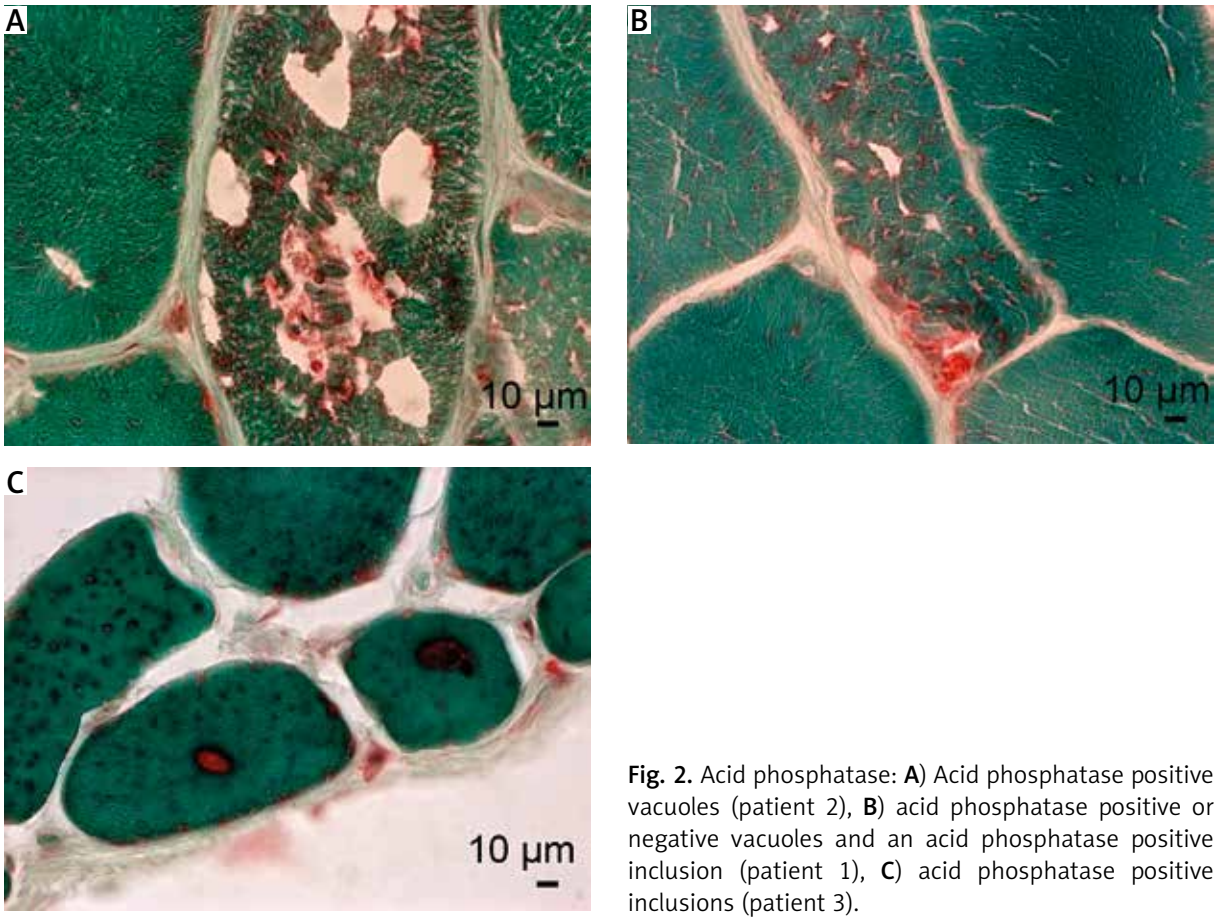


Fig. 2. Acid phosphatase: **A**) Acid phosphatase positive vacuoles (patient 2), **B**) acid phosphatase positive or negative vacuoles and an acid phosphatase positive inclusion (patient 1), **C**) acid phosphatase positive inclusions (patient 3).

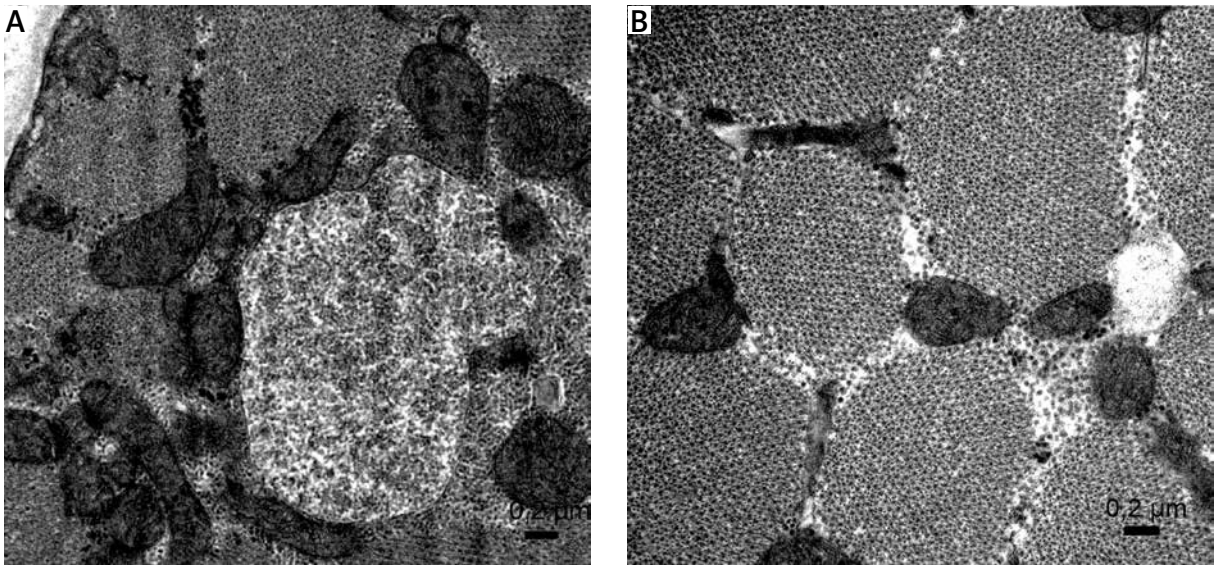


Fig. 3. Electronic microscopy: **A**) lysosomal accumulation of glycogen (patient 2), **B**) extra-lysosomal accumulation of glycogen on electronic microscopy (patient 4).

rimmed vacuoles would be artifacts formed during the coloring process: the rimmed vacuoles would correspond to clusters of autophagic vacuoles coming off the glass slide and regrouped in myofibrils nearby.

Although Nishino reported that some histological aspects observed in Pompe disease look like rimmed vacuoles [11], he does not include this disease in the list of numerous inherited diseases characterized by this histological abnormality (hereditary inclusion body myopathy, distal myopathy with rimmed vacuoles, limb-girdle muscular dystrophy 2G, inclusion body myopathy 3, limb-girdle muscular dystrophy 1A, oculopharyngeal muscular dystrophy, desmin myopathy, desmin-related myopathy, tibial muscular dystrophy) [12].

To our knowledge, Schoser *et al*. [15] are the only ones to have previously reported aspects of "rimmed vacuoles" in muscle biopsies of cases of the adult form of Pompe disease; this was observed in rare biopsies in their cohort of 38 patients.

In our study, biopsies of all patients with late-onset Pompe disease had genuine rimmed vacuoles even when glycogen deposits were moderate or minimal in electronic microscopy (patients 1, 2, 4).

Based on clinicopathological correlations (ptosis and rimmed vacuoles), our case 2 was initially misdiagnosed with oculopharyngeal dystrophy. Our study highlights that it is thus important for a pathologist to include the Pompe disease in the list of diseases characterized by rimmed vacuoles, especially since effective treatment is available for this disease of acid maltase deficiency.

Conclusions

The presence of both acid phosphatase positive inclusions and rimmed vacuoles in muscle biopsies should thus suggest a diagnosis of Pompe disease in case of clinically atypical forms, and prompt the clinician to make measurement of enzyme activity and analysis in molecular biology to confirm the diagnosis. These new diagnostic screening markers of Pompe disease should, however, be validated on a larger number of cases.

Acknowledgements

The authors are grateful to Chantal Chavoix, Caen University Hospital, for her help in editing the manuscript.

Disclosure

Authors report no conflict of interest.

References

1. American Association of Neuromuscular & Electrodiagnostic Medicine. Diagnostic criteria for late-onset (childhood and adult) Pompe disease. Muscle Nerve 2009; 40: 149-160.
2. Dimachkie MM, Barohn RJ. Inclusion body myositis. Curr Neurol Neurosci Rep 2013; 13: 321.
3. DiMauro S, Spiegel R. Progress and problems in muscle glycogenoses. Acta Myol 2011; 30: 96-102.
4. Feeney EJ, Austin S, Chien Y-H, Mandel H, Schoser B, Prater S, Hwu WL, Ralston E, Kishnani PS1, Raben N. The value of muscle biopsies in Pompe disease: identifying lipofuscin inclusions in juvenile- and adult-onset patients. Acta Neuropathol Commun 2014; 2: 2.
5. Hagemans MLC, Winkel LPF, Van Doorn PA, Hop WJ, Loonen MC, Reuser AJ, Van der Ploeg AT. Clinical manifestation and natural course of late-onset Pompe's disease in 54 Dutch patients. Brain J Neurol 2005; 128 (Pt 3): 671-677.
6. Hobson-Webb LD, Proia AD, Thurberg BL, Banugaria S, Prater SN, Kishnani PS. Autopsy findings in late-onset Pompe disease: a case report and systematic review of the literature. Mol Genet Metab 2012; 106: 462-469.
7. Laforêt P, Laloui K, Granger B, Hamroun D, Taouagh N, Hogrel JY, Orlikowski D, Bouhour F, Lacour A, Salort-Campana E, Penisson-Besnier I, Sacconi S, Zagnoli F, Chapon F, Eymard B, Desnuelle C, Pouget J. The French Pompe registry. Baseline characteristics of a cohort of 126 patients with adult Pompe disease. Rev Neurol (Paris) 2013; 169: 595-602.
8. Lewandowska E, Wierzba-Bobrowicz T, Rola R, Modzelewska J, Stepień T, Lugowska A, Pasennik E, Ryglewicz D. Pathology of skeletal muscle cells in adult-onset glycogenosis type II (Pompe disease): ultrastructural study. Folia Neuropathol 2008; 46: 123-133.
9. Lu H, Luan X, Yuan Y, Dong M, Dong M, Sun W, Yan C. The clinical and myopathological features of oculopharyngodistal myopathy in a Chinese family. Neuropathology 2008; 28: 599-603.
10. Malicdan MCV, Noguchi S, Nishino I. Autophagy in a mouse model of distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy. Autophagy 2007; 3: 396-398.
11. Malicdan MCV, Nishino I. Autophagy in lysosomal myopathies. Brain Pathol 2012; 22: 82-88.
12. Nishino I. Autophagic vacuolar myopathies. Curr Neurol Neurosci Rep 2003; 3: 64-69.
13. Saux A, Laforet P, Pagès AM, Figarella-Branger D, Pellissier JF, Pagès M, Labauge P. A retrospective study of six patients with late-onset Pompe disease. Rev Neurol (Paris) 2008; 164: 336-342.
14. Schneider I, Hanisch F, Müller T, Schmidt B, Zierz S. Respiratory function in late-onset Pompe disease patients receiving long-term enzyme replacement therapy for more than 48 months. Wien Med Wochenschr 2013; 163: 40-44.
15. Schoser BGH, Müller-Höcker J, Horvath R, Gempel K, Pongratz D, Lochmüller H, Müller-Felber W. Adult-onset glycogen stor-

age disease type 2: clinico-pathological phenotype revisited. Neuropathol Appl Neurobiol 2007; 33: 544-559.
16. Sharma MC, Schultze C, von Moers A, Stoltenburg-Didinger G, Shin YS, Podskarbi T, Isenhardt K, Tews DS, Goebel HH. Delayed or late-onset type II glycogenosis with globular inclusions. Acta Neuropathol 2005; 110: 151-157.
17. Steps forward in Pompe Disease. Abstracts of the 4th European Symposium. November 19-20, 2010. London, United Kingdom. Clin Ther 2011; 33 (6 Suppl 1): S1-40.
18. Tsuburaya RS, Monma K, Oya Y, Nakayama T, Fukuda T, Sugie H, Hayashi YK, Nonaka I, Nishino I. Acid phosphatase-positive globular inclusions is a good diagnostic marker for two patients with adult-onset Pompe disease lacking disease specific pathology. Neuromuscul Disord 2012; 22: 389-393.

DOI: 10.5114/fn.2016.62540

Joint Conference

The 13th International Symposium

MOLECULAR BASIS OF PATHOLOGY AND THERAPY IN NEUROLOGICAL DISORDERS

The 4th International Conference

STEM CELLS: THERAPEUTIC OUTLOOK FOR CENTRAL NERVOUS SYSTEM DISORDERS

November 17-18, 2016

Warsaw, Poland

ORGANIZERS

Mossakowski Medical Research Centre, Polish Academy of Sciences
Cell Therapy Team of the Central Nervous System Diseases –
Committee of Neurological Sciences, Polish Academy of Sciences
Committee of Physiology and Pharmacology Sciences, Polish Academy of Sciences

SCIENTIFIC COMMITTEE

Elzbieta Salinska
Leonora Buzanska
Krystyna Domanska-Janik
Anna Sarnowska
Jerzy Lazarewicz
Magdalena Zielinska
Monika Szeliga
Malgorzata Beresewicz
Lidia Struzynska
Barbara Zablocka
Teresa Zalewska

The communications presented at the Symposium are printed without alterations from the manuscripts submitted by the authors, who bear the full responsibility for their form and content.

SPONSORS:

Poster session I

|A1|

Comparison of gene transcription and depressive-like symptoms following chronic morphine and dexamethasone administration in mice

Barut J., Skupio U., Marut L., Przewlocki P.

Department of Molecular Neuropharmacology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Summary of the objectives: The molecular mechanism underlying opiate withdrawal-induced depression remains unclear. One consistently reported dysfunction in depression is the dysregulation of the stress system, the hypothalamus-pituitary-adrenal axis (HPA) which is associated with alterations in glucocorticoid receptors (GRs) function. Hippocampus, a part of limbic system has important role in regulating the HPA stress response. Therefore, we utilized a mouse model of repeated morphine and GR agonist dexamethasone administration to examine the consequences of prolonged withdrawal-induced depressive-like behaviors and to compare and examine the underlying mechanism of action of these drugs.

Methodology: C57BL/6J mice were injected twice a day for 3 weeks with morphine (MOR, increasing doses, 20-100 mg/kg i.p.), once a day with dexamethasone (DEX, 4 mg/kg i.p.) or saline (SAL, 10 ml/kg i.p.). All groups of animals were left for 3 weeks to spontaneously withdraw without injection and depressive-like behaviors were evaluated. We used real-time PCR to map transcription in the hippocampus of mouse brain undergoing MOR and DEX treatment and followed by the 21 days of abstinence.

Key results: MOR- as well as DEX-abstinent animals exhibited a significant depression symptoms. Our analysis found that mRNA expression of GR (Nr3c1) was decreased in hippocampus of MOR- and DEX-treated as well as MOR- and DEX-withdrawn mice. ANOVA tests indicated significant differences between MOR and DEX-treated in immediate-early genes expression (c-Fos, Arc, Npas4 and Bdnf). Previous analysis revealed that MOR affects expression of CAMK family genes. We noticed that MOR and DEX treatment significantly modified levels of mRNA Camk1g, one of the characteristic gene of the structure of hippocampus.

Major conclusions: Acute administration of MOR and DEX initially affects glucocorticoid pathway in the hippocampus in a similar manner. In difference, the prolonged drugs administration and abstinence regulates the molecular pathways differentially. Thus, differential molecular mechanism appears to be involved in depression evoked by opioid withdrawal and chronic glucocorticoids in hippocampus.

Acknowledgments: Funding for this study was provided by Polish National Science Centre. Grant: 2013/08/A/NZ3/00848.

|A2|

Beta(1)-adrenergic receptor blockade during chronic restraint stress modulates the expression of apoptotic signaling-related genes in rat hippocampus

Bielawski A., Zelek-Molik A., Kowalska M., Wiatrowska K., Nalepa I.

Department of Brain Biochemistry, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Stress may impair the structure and activity of the hippocampus (HIP) and play a role in the pathology of stress related psychiatric disorders. The noradrenaline released during stress is known to stimulate the beta adrenergic receptors (beta-AR) highly expressed in HIP. Also, apoptotic and degenerative changes in the hippocampus were reported in animal stress models. It has been shown that in the control of apoptosis many intracellular pathways are implicated and the beta-AR as well. We aimed to evaluate the stress induced effects on expression of the apoptotic signaling-related genes in the HIP and to assess whether they can be affected by beta(1)-AR blockade.

Male Wistar rats underwent the chronic restraint stress procedure applied for 3 hours daily, for 14 days. During the last 7 days rats were treated with beta-AR blocker betaxolol (5 mg/kg p.o.) given immediately after daily stress. Next day after a completion of stress procedure, the rats were decapitated and their HIPs were dissected. Then, the real-time PCR reaction with TaqMan Low Density Arrays (TLDA) was used to study the pattern of gene expression and identification of particular genes under stress reaction.

Two-way ANOVA analysis showed that chronic restraint stress increases the expression of Ikbkg mRNA, and treatment with betaxolol enhances this stress effect. Moreover, clustering analysis revealed the existence of two groups of apoptotic-related genes – down- and up-regulated by chronic restraint stress. The former consists of Casp7, Ripk3, Tnfsf10, and the latter – Dffa, Bcl2l13, Daxx. Betaxolol augmented changes caused by stress in both groups.

The results indicate that chronic restraint stress induced changes in mRNA expression of several pro-apoptotic genes belonging to various pathways involved in a control of apoptotic process. Among them the stress increased Ikbkg gene encodes for regulatory subunit of the inhibitor of kappaB kinase complex which activates NF-kappaB. This transcription factor, critical during inflammation, is also an important regulator of synaptic plasticity. Furthermore, the enhancement by beta(1)-AR blockade of the stress-induced effects on genes' expression suggests that the regulation of these genes occurs via the protein kinase A dependent manner.

Supported by statutory funds of the Institute of Pharmacology, Polish Academy of Sciences.

|A3|

Group II metabotropic glutamate receptors activation reduce apoptotic processes evoked by hypoxia-ischemia in 7-day old rat pups

Bratek E., Ziembowicz P., Salinska E.

Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Hypoxic-ischemic encephalopathy (HIE) results in permanent damage of central nervous system that may result in neonatal death or developmental disorders. 20-30% of infants with HIE die in the neonatal period, and 33-50% of survivors demonstrate permanent neurodevelopmental abnormalities and mental retardation. It was shown recently that activation of group II metabotropic glutamate receptors (mGluR2/3) in a short time after ischemic insult may results in neuroprotection but the exact mechanism of this effect is not clear. The aim of present study was to investigate whether mGluR2/3 activation after hypoxia-ischemia reduces brain damage and if the inhibition of apoptotic processes is one of the involved mechanisms.

We used an animal model of hypoxia-ischemia (H-I) on 7-day old rat pups. Animals were anesthetized and the left common carotid artery was isolated, double – ligated and then cut between the ligatures. After completion of the surgical procedure the pups were subjected to hypoxia (7.4% oxygen in nitrogen for 75 min at 35°C). Control pups were sham-operated (anaesthetized and left c.c.a. dissected, but not ligated). Animals were injected intraperitoneally with specific mGluR2 (LY 379268) or mGluR3 (NAAG) agonists 1 h or 6 h after H-I (5 mg/kg of body weight). The weight deficit of the ischemic brain hemisphere was measured and the expression of pro-apoptotic and anti-apoptotic factors (Bax, Bcl-2, HTR/OMI) was examined. The damage in the hippocampal CA1 region was examined by Cresyl violet (CV) staining.

Our results show that application of mGluR2/3 agonists after H-I results in neuroprotection. Both applied agonists decreased brain tissue weight loss in ischemic hemisphere at both times of application (from 40% in H-I to 15-20% in treated). Histological examination of the brain tissue showed that both mGluR2/3 antagonists applied 1 h or 6 h after H-I decreased the damage of neuronal cells and the disorganization of CA1 region of hippocampus. Our results show also that both mGluR2/3 antagonists applied 1 h or 6 h after HI significantly reduced number of TUNEL-positive cells in ipsilateral hemispheres observed after untreated HI. The activities of pro-apoptotic caspase-3 and -9 after HI insult increased significantly in comparison to control. The injection of either of mGluR2/3 agonists 1 h or 6 h after HI significantly reduced the activities of both caspases.

Agonist of mGluR2/3 applied 1 h or 6 h after H-I decreased expression of pro-apoptotic factors Bax and HTR/OMI and increased expression of anti-apoptotic Bcl-2 in the ischemic brain hemisphere compared to H-I.

Conclusions: The results show that activation of mGluR2 or mGluR3 in a short time after H-I insult triggered neuroprotective mechanisms and reduced apoptotic processes initiated by HI in developing brain.

This work was made under MMRC-KNOW 2013-2017 project (KNOW-14).

|A4|

Functional inhibitors of acid sphingomyelinase as a new therapeutic target for depression

Brodowicz J.[1,2], Filip M.[2]

[1]Faculty of Pharmacy, Jagiellonian University, Medical College, Cracow, Poland
[2]Laboratory of Drug Addiction Pharmacology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Background and aim: Dysregulation of the ceramide metabolism (e.g., by the acid sphingomyelinase) has been proposed as an important factor in the pathogenesis of depressive disorders. Moreover, some antidepressant drugs func-

tion as the acid sphingomyelinase inhibitors and decrease ceramide levels in the rat hippocampus. The aim of this study was to analyze effects of antidepressants having different mechanisms of action and chemical structures (imipramine, tianeptine, escitalopram) as well as the substance showing antidepressant activity in preclinical research (N-acetylcysteine) on several components of ceramide metabolism in the rat hippocampus and cerebellum.

Material and methods: Male Wistar rats received imipramine (IMI, 15 mg/kg), tianeptine (TIA, 10 mg/kg), escitalopram (ESC, 10 mg/kg), N-acetylcysteine (NAC, 100 mg/kg) or corresponding vehicles acutely or chronically (for 14 days). Twenty four hours after the last injection the animals were decapitated. Brain structures were analyzed using Western Blot.

Results: We found significant increases in the synthase ceramide 2 levels after acute administration of IMI or TIA in the hippocampus as well as after chronic administration of IMI in the cerebellum. Acute and chronic administration of IMI resulted in a significant increase in the level of the ceramide synthase 4 in the cerebellum and hippocampus, respectively. On the other hand, an acute administration of TIA and the chronic administration of NAC induced a significant decrease in the synthase ceramide 4 protein expression in the hippocampus. After chronic administration of IMI and ESC, a significant increase in the synthase ceramide 5 levels in the hippocampus was found; the increase in the latter enzyme was noted for the acute administration of NAC in the cerebellum. At the end, we report the increase in the hippocampal level of neutral sphingomyelinase after chronic administration of TIA and the decrease in the cerebellar alkaline sphingomyelinase after chronic treatment with IMI.

Conclusions: Our findings indicate that different antidepressant drugs alter the expression of number of enzymes in the ceramide metabolism what may further highlight the role of this sphingolipid in the pathophysiology of depression.

|A5|

The effect of acute normobaric hypoxia on circulating BDNF during exercise to volitional exhaustion in young sedentary man and elite endurance-trained athletes

Chalimoniuk M.[1], Czuba M.[2], Smoll E.[3], Langfort J.[4]

[1]Department of Cellular Signaling, Mossakowski Medical Research Centre, Warsaw, Poland
[2]Department of Sports Theory, The Jerzy Kukuczka Academy of Physical Education, Katowice, Poland
[3]Department of Physiology, The Jerzy Kukuczka Academy of Physical Education, Katowice, Poland
[4]Department of Nutrition and Supplementation, The Jerzy Kukuczka Academy of Physical Education, Katowice, Poland

mchalimoniuk@imdik.pan.pl

During hypoxia exercise performance involving large muscle groups is considerably lower as compared with normoxia. This phenomenon is attributed to both muscle fatigue and so-called central fatigue. Most recent data suggest that associated with central fatigue hypoxia-related reduction of central motor output precedes the development of peripheral muscle fatigue. It has been suggested that an essential gauge role in these phenomena is played by brain-derived neurotrophic factor (BDNF). If so, BDNF release should have been differed between sedentary and endurance trained subjects during exercise, because well-trained athletes are more resistant to exercise induced fatigue. To test this hypothesis 10 healthy young males and 10 elite cyclist performed incremental exercise to volitional exhaustion (EVE) at normoxic and hypoxic conditions. The normobaric hypoxic conditions were suited to 3000 m altitude (14.7% O_2). An impact of hypoxia on participants' organism was investigated by measurement of serum EPO that was increased during EVE in hypoxic conditions in both participants groups. In normoxic conditions an increase in serum BDNF during EVE was observed only in elite endurance trained cyclists whilst in hypoxic conditions elevated level in serum BDNF was seen in both groups. This finding suggests that exercise in hypoxic conditions could stand as a more potent and effective strategy for increasing circulating BDNF than exercise performed in normoxic conditions.

This work was supported by the grant No. 2013/09/B/NZ7/00726 from the National Science Centre of Poland.

|A6|

Endurance bout of exercise upregulates dopamine metabolism in nigrostriatal system of rats

Chalimoniuk M.[1], Blugental N.[1,4], Orzel K.[1,5], Langfort J.[2,3]

[1]Department of Cellular Signaling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Experimental Pharmacology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[3]Department of Nutrition and Supplementation, The Jerzy Kukuczka Academy of Physical Education, Katowice, Poland
[4]Department of Animal Physiology, Faculty of Biology, University of Warsaw, Poland
[5]Faculty of Agriculture and Biology, Warsaw University of Life Sciences, Warsaw, Poland

The physical activity status of the organism impacts the function of the nervous system. Among effects of enhanced physical activity, secretion of neurotransmitters, especially monoamines, have been linked to the exercise-induced neuronal adaptation/plasticity. Interplay between exercise and monoamines was initially derived from the "Central Fatigue Hypothesis", in which increased brain 5-HT release was found to be associated with central fatigue. Most recent data suggest a possible role of dopaminergic pathway located within nigrostratial system in the control of locomotion. We hypothesized this system can be also stimulated by a bout endurance exercise of moderate intensity. Rats were running on the treadmill (0° inclination) at 24 m/min to exhaustion. Rats were killed immediately after exercise and striatum and midbrain were quickly isolated on ice-cold glass Petri dish and estimation of TH mRNA and protein level, activity of MAOB and levels of DA as well as its metabolites concentrations in striatum and midbrain were performed. In the present study, the bout of endurance exercise of moderate intensity resulted in increased TH mRNA and protein level as well as MAOB activity in the striatum and midbrain. These results suggest that favorable effects of endurance exercise are related to the enhanced dopamine synthesis and metabolism in the nigrostriatal system.

Supported by the NCN grant #2011/01/B/ZN5/01397.

|A7|

PARP-1 and its role in transcription of mitochondrial respiratory complexes and enzymes of antioxidative defence under basal conditions and in Amyloid Beta toxicity

Cieslik M.[1], Baranski D.[1], Wencel P.[2], Czapski G.A.[1], Strosznajder R.P.[2], Strosznajder J.B.[1]

[1]Department of Cellular Signalling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Neurosurgery, Laboratory of Preclinical Research and Environmental Agents, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
mcieslik@imdik.pan.pl

Poly(ADP-ribose) polymerase-1 (PARP-1) is the major member of PARPs family mainly responsible for a post-translational modification of transcription factors, affecting expression of many nuclear and mitochondrial proteins. PARP-1 plays also an important role in DNA repair and in energy metabolism regulating cells survival and death. Overactivation of PARP-1 by excessive genotoxic stress may lead to cell death in Alzheimer's disease (AD). Our previous data indicated beneficial effects of PARP-1 inhibition in neurodegenerative disorders. The aim of this study was to investigate the role of PARP-1 in regulation of gene expression of enzymes responsible for function of mitochondrial respiratory chain complexes and for antioxidative defense in resting pheochromocytoma PC12 cells and in conditions of Amyloid Beta 1-42 (AB) toxicity.

The study was carried out using PC12 cells treated with PARP-1 inhibitor PJ34 (20 μM) or with AB oligomers (ABO, 1 μM) for 24 h. Quantitative RT-PCR as well as biochemical, immunochemical, spectrofluorometric and flow-cytometric methods were applied.

Our data indicate that pharmacological inhibition of PARP-1 in PC12 cells by PJ34 enhances transcription of enzymes responsible for mitochondrial metabolism. PJ34 increases the mRNA level of subunit of NADH:ubiquinone oxidoreductase (MT-ND1) of complex I and has similar stimulatory effect on expression of gene for subunit of succinate dehydrogenase (SDHA) of complex II. Additionally, PJ34 augments transcription of cytochrome b (MT-CYB) of complex III and subunit of cytochrome c oxidase (MT-CO1) of complex IV. These changes may enhance mitochondrial activity leading to higher production of free radicals. Analysis of enzymes involved in antioxidative defense indicates downregulation of mitochondrial superoxide dismutase (SOD2) and glutathione-disulfide reductase (GSR). ABO inhibit expression of SDHA, SOD2, and reduce

mitochondrial membrane potential (MMP) and cell viability. PJ34 has no protective effect on suppression of MMP and cell viability by ABO. Our data indicate that inhibition of PARP-1 enhances the expression of genes involved in mitochondrial function; however, has no protective effect on MMP and PC12 survival affected by ABO. These results show the complex role of PARP-1 in cell metabolism and cell fate and suggest dependence on cell type and experimental conditions.

Supported by The National Science Centre (http://www.ncn.gov.pl) Grant 2013/09/B/NZ3/01350.

|A8|

Maternal immune activation alters the synaptic protein level and mTOR signaling pathway in rat offspring

Cieslik M.[1], Gassowska M.[1], Jesko H.[1], Dominiak A.[2], Filipkowski R.[3], Boguszewski P.M.[4], Adamczyk A.[1]

[1]Department of Cellular Signaling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Drug Bioanalysis and Analysis, Medical University of Warsaw, Warsaw, Poland
[3]Behavior and Metabolism Research Laboratory, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[4]Department of Neurophysiology, Laboratory of Limbic System Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Autism spectrum disorders (ASDs) are complex neurodevelopmental brain diseases characterized by deficits in social interaction, language and stereotyped behaviours. Recent discoveries of single mutations in genes coding synaptic proteins in affected individuals suggested the synapse as a possible site of autism origin. Maternal immune activation (MIA) during pregnancy is a risk factor for autism in the offspring and is commonly used as animal model of ASD. Infections during pregnancy activate the mother's immune system and alter the fetal environment, and in consequence can affect synaptic function and behaviour in the offspring. However, the molecular link between infection-induced altered fetal development and risk for ASD are still unclear. In this study we investigated behavioural changes, synaptic protein expression as well as Akt-mTOR pathway protein level in the offspring of pregnant Wistar rats given an intraperitoneal (0.10 mg/kg) injection of lipopolysaccharide on gestational day 9.5. Our results indicated that communication (expressed by ultrasonic vocalizations, USVs) of rat pups born to MIA mothers compared to pups born to saline-injected mothers was impaired. Analysis of USV of 9-11-day-old animals showed a longer mean time vocalization in pups from MIA mothers with significantly lower frequency of USV. Moreover, the results showed no bedding preference in MIA offspring at post-natal day 15 compared to control rats, indicating the impairment of need being in proximity of the mother. Along with the behavioural changes MIA induced presynaptic protein alterations in adolescent rat offspring including decrease in the level of synaptobrevin and syntaxin-1, the key components of SNARE complex. However, the higher level of synapsin was observed in brain cortex and hippocampus. Together with presynaptic protein changes MIA induced reduction in PSD-95 in the cerebral cortex and hippocampus and down-regulation of SHANK 1-3. Moreover, alteration in the protein level of phospho-Akt, and 4E-BP1 was found in MIA subjects. It is possible that alterations of Akt-mTOR pathway result from aberrant synthesis of postsynaptic density proteins as supported by decreased level of SHANK and PSD-95. The altered synthesis of these proteins would generate changes in molecular and structural aspects of synaptic plasticity, contributing to ASD-like behaviours.

Supported by the statutory theme 8.

|A9|

Upregulation of TNF-α and IL-6 in serum and cerebrospinal fluid of patients with ALS

Czarzasta J.[1], Wojtacha P.[1], Jezierska-Wozniak K.[2,3], Siwek T.[3], Wojtkiewicz J.[1,2,4], Maksymowicz W.[3]

[1]Department of Pathophysiology, University of Warmia and Mazury, Olsztyn, Poland
[2]Laboratory of Regenerative Medicine, University of Warmia and Mazury, Olsztyn, Poland
[3]Department of Neurology and Neurosurgery, Faculty of Medical Sciences, University of Warmia and Mazury, Olsztyn, Poland
[4]Foundation for Nerve Cells Regeneration, Olsztyn, Poland

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder without effective cure. The involvement of inflammation in the pathogenesis of ALS, is increasingly recognized but still not fully understood. Therefore, the purpose of this study was to investigate the levels of inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-4 in serum and

cerebrospinal fluid (CSF) of ALS patients. The study was approved by the Local Ethic Committee of University of Warmia and Mazury in Olsztyn, Poland. The diagnosis of ALS and the evaluation of the revised ALS functional rating scale (ALSFRS-R) score were performed for each ALS patient by neurologist. Blood and CSF samples were drawn from 10 ALS patients and 10 patients with other non-inflammatory neurological disorders (NND) served as a control group. Enzyme-like immunosorbent assay (ELISA) was used to check serum and CSF levels of TNF-α, IL-6 and IL-4. Unpaired, independent 2-tailed Student *t*-test was used and the statistical data were expressed as the mean ± SEM, statistical significance was defined as a *p*-values below 0.05.

The concentrations of TNF-α and IL-6 in serum and CSF of ALS patients were greater than in NND patients. There was no statistical difference in serum IL-4 level between studied groups, however, in the ALS patients the amount of this cytokine was lower. In the CSF, the level of IL-4 was not detectable. Our study shows the upregulation of TNF-α and IL-6 in serum and cerebrospinal fluid of ALS patients, which suggests the importance of these inflammatory factors in the course of the disease.

|A10|

Tetrahydrocarbazoles stabilize elevated SOCE in a Huntington's disease model, MSNs from YAC128 mice overexpressing huntingtin-associated protein 1 isoform A

Czeredys M., Maciag F., Kuznicki J.

International Institute of Molecular and Cell Biology, Warsaw, Poland

Store-operated Ca^{2+} entry (SOCE) is one of the mechanisms that regulate Ca^{2+} homeostasis and is enhanced in Huntington's disease (HD). However, it is still unknown how mutated huntingtin affects SOCE and there is no effective treatment of HD. We previously showed that huntingtin associated protein 1 (HAP1) is up-regulated in the striatum of HD model, YAC128 mice. We also found that selected tetrahydrocarbazoles stabilize the ER Ca^{2+} release in cellular Alzheimer's disease model. The aim of this work was to investigate the role of HAP1 protein in SOCE dysregulation and check the effect of tetrahydrocarbazoles on the ER Ca^{2+} release and SOCE as well as cell death in YAC128 medium spiny neurons (MSNs). Single cell Ca^{2+} imaging, gene silencing and overexpression as well as cell death and mitochondrial membrane potential assays were used for this purpose. We observed that HAP1 isoform A overexpression decreases ionomycin induced ER Ca^{2+} release and enhances SOCE, whereas its silencing attenuates SOCE and decreases ER Ca^{2+} release induced by DHPG, an mGluR1/5 receptor agonist. In HD MSNs overexpressing HAP1A we found that certain tetrahydrocarbazoles have a stabilizing effect on elevated SOCE, however, no effect on ER Ca^{2+} release was observed. Moreover, we found that some of tetrahydrocarbazoles increase mitochondrial membrane potential, but they are not able to stabilize glutamate induced cell death in HD model. We conclude that HAP1A increases SOCE in HD MSNs by IP3R activation and tetrahydrocarbazoles exhibit stabilizing effect on the disturbed Ca^{2+} homeostasis in HD model.

|A11|

The involvement of Sp1 in transcriptional regulation of the glutamine transporter SN1 in cultured mouse cortical astrocytes treated with ammonia

Dabrowska K., Obara-Michlewska M., Albrecht J., Zielinska M.

Department of Neurotoxicology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Ammonia neurotoxicity plays a key role in the pathogenesis of hepatic encephalopathy (HE). Astrocytes are the only compartment in the brain where ammonia detoxification triggers intracellular glutamine (Gln) accumulation. Gln efflux from astrocytes is mediated by a system N transporter, SN1 (SNAT3) that demonstrates the ability to change transport direction with changing extracellular pH (pHe) and transmembrane gradients of amino acids (Chaudhry *et al*., 1999; Broër *et al*., 2002). In addition, the gene coding SN1 (SNAT3) has a putative pH responsive element in the 3'-UTR (Solbu *et al*., 2005). Literature data suggest that during ammonia-induced metabolic acidosis in mouse kidney, SN1 is upregulated by interactions with the specificity protein 1 (Sp1) transcription factor (Balkrishna *et al*., 2013). In turn, Sp1 presents a tendency toward increase in cultured rat astrocytes treated with 5 mM ammonia (Bodega *et al*., 2006). However, the specific regulatory role of Sp1 in transcriptional regulation of SN1 in astrocytes treated with ammonia and its relation to ammonia-induced changes in pH was not analyzed so far. The latter hypothesis was tested in the present study.

Using real-time qPCR we measured SN1 and Sp1 mRNA level in cultured mouse cortical astrocytes treated with 5 mM ammonium chloride for 24 h and to mild (pH 6.8) acidosis. Simultaneously, the impact of ammonia and acid incubation on intracellular (pHi) and pHe was evaluated. Sp1 transcription factor silencing was performed using siRNA technology. pHi was measured using fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetomethyl ester (BCECF-AM).

Sp1 mRNA level showed tendency toward increase (similarly to previous study on rat cortical astrocytes) in ammonia-treated astrocytes, whereas acidic media reduced Sp1 mRNA level. Ammonia did not alter SN1 mRNA level, but acidic media did. Neither treatment changed pHi, but pHe was elevated after ammonia treatment. Sp1 silencing in ammonia treated astrocytes decreased SN1 mRNA level while in acidic medium a return of SN1 mRNA level was observed.

In conclusion, our study demonstrates that SN1 mRNA expression is dependent on the presence of Sp1 transcription factor, suggesting that Sp1 is an enhancer of SN1 expression. Moreover, a decrease of pHe downregulates SN1 mRNA expression.

Supported by NCBR "CORE" Pol-Nor/196190/26/2013.

|A12|

Is DCF test a suitable tool for the evaluation of tetrabromobisphenol A-induced oxidative stress in cultured neurons?

Diamandakis D., Zieminska E., Lenart J., Lazarewicz J.W.

Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Tetrabromobisphenol A (TBBPA) belongs to brominated flame retardants exhibiting cyto(neuro)toxic properties in cell culture models. The role of oxidative stress in TBBPA-induced cytotoxicity has been well established, mainly based on the results of DCF assay demonstrating enhanced ROS production in TBBPA-treated cells. However, according to recent reports, in cell-free solutions TBBPA chemically converts a parent compound DCFH-DA into fluorescent DCF, which casts doubt on earlier reports showing TBBPA-induced oxidative stress in cells. The aim of the present study was to asses reliability of DCF assay in evaluating TBBPA-induced oxidative stress in primary cultures of rat cerebellar granule cells (CGC). The experimental approach involved measurements in CGC and in cell-free solutions, using a standard plate reader, of the effects of 10 and 25 μM TBBPA on fluorescence of DCF. The level of oxidative stress in CGC challenged with TBBPA, which was also assessed by measuring GSH content and catalase activity, was modulated pharmacologically using NMDA and ryanodine receptor antagonists, 0.5 μM MK-801 and 200 μM ryanodine with 2.5 μM bastadin 12, respectively. They are known to have no intrinsic antiradical properties but inhibit TBBPA-induced calcium transients in CGC. The experiments on CGC demonstrated TBBPA-induced, concentration-dependent rise in DCF fluorescence which was accompanied by a concomitant decrease in GSH content and catalase activity. These phenomena were inhibited by NMDA and ryanodine receptor antagonists, which were by themselves ineffective in control CGC loaded with DCFH-DA. The results of experiments in cell-free system confirmed that TBBPA potentiates fluorescence of cell-free DCFH-DA solution, but this effect was not inhibited by NMDA and ryanodine receptor antagonists and was even enhanced by bastadin 12. Moreover TBBPA decreased fluorescence of rhodamine 123 solution, while in CGC loaded with this probe it enhanced fluorescence. Thus, the results of biological experiments in accordance indicate, that TBBPA induces oxidative stress in CGC, and DCF test in CGC reflects enhanced ROS production under these conditions. Instead, the effects observed in the cell-free DCFH-DA and rhodamine 123 solutions are irrelevant to data from the living cells. The presentation will show provisional explanation for these discrepancies.

Supported by NCN grant no. 2012/05/B/NZ7/03225.

|A13|

Perinatal exposure to lead alters the synaptic structure and the expression of key synaptic protein in Wistar rats

Gassowska M.[1], Baranowska-Bosiacka I.[2], Moczydlowska J.[1], Frontczak-Baniewicz M.[3], Gewartowska M.[3], Struzynska L.[4], Chlubek D.[2], Adamczyk A.[1]

[1]Department of Cellular Signaling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Biochemistry and Medical Chemistry, Pomeranian Medical University, Szczecin, Poland
[3]Electron Microscopy Platform, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[4]Laboratory of Pathoneurochemistry, Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

magy80@gmail.com

The heavy metal lead (Pb) is an abundantly existing environmental toxicant for the development of central nervous system. Prenatal lead exposure has negative impacts on many neurodevelopmental processes including synaptogenesis, apoptosis and causes abnormalities in learning, and cognitive functions in the offspring. However, up till now there is no exact mechanism explaining molecular events leading to synaptic endings impairments. The aim of the present study was to investigate the effect of perinatal exposure to low dose of Pb (Pb concentrations in whole blood below 10 µg/dl) on the synaptic structure and the synaptic proteins expression in the developing rat brain. Furthermore, the brain-derived neurotrophic factor (BDNF) level was analyzed. Lead (0.1% PbAc) was administrated to pregnant Wistar rats via drinking water (Pb-group) from the first day of gestation until weaning of the offspring. Pups were weaned at postnatal day 21 and then until postnatal day 28 received only drinking water. At the end of experiments, 28-day old pups were sacrificed and the ultrastructural changes as well as expression of presynaptic (VAMP1/2, Synaptophysin, Synaptotagmin-1, SNAP25, Syntaxin-1) and postsynaptic (PSD95) proteins, and BDNF level were analyzed in forebrain cortex, cerebellum and hippocampus. We showed that perinatal Pb exposure promotes pathological changes in synapses including nerve endings swelling, blurred and thickened synaptic cleft structure as well as enhanced density of synaptic vesicles clustering in the presynaptic area. Moreover, synaptic mitochondria were elongated, swollen or shrunken in Pb-group. Together with ultrastructural changes we observed lowering of the level of Synaptotagmin-1 in cerebellum, SNAP25 in hippocampus and Syntaxin-1 in cerebellum and hippocampus. In addition, in cerebellum Synaptophysin level was increased. The expression of PSD95 was significantly reduced in this structure and in forebrain cortex, but increased in hippocampus. These changes are accompanied by lower level of BDNF in all brain structures from Pb-group. In summary, we found that perinatal Pb exposure affected the synaptic structure and the key synaptic proteins expression, that could impair synaptic plasticity as well as the learning and memory processes in the offspring.

Supported from MMRC statutory theme 8 and statutory budget of PUM in Szczecin.

|A14|

AMPA receptors are involved in STIM-dependent Store-Operated Calcium Entry in rat primary cortical neurons

Gruszczynska-Biegala J., Sladowska M., Kuznicki J.

Laboratory of Neurodegeneration, International Institute of Molecular and Cell Biology, Warsaw, Poland

joannag@iimcb.gov.pl

Store-Operated Calcium Entry (SOCE) is a process, which leads to refilling of endoplasmic reticulum (ER) with calcium ions (Ca^{2+}) after their release into the cytoplasm. The interaction between ER-located proteins (STIM1, STIM2) and plasma membrane (PM)-located Ca^{2+} channel-forming protein (ORAI1) mediates the formation of complexes and underlies SOCE in non-excitable cells. Recent studies have recognized the importance of SOCE also in neurons and found complex relationship between STIM proteins and neuronal Ca^{2+} channels, but its molecular mechanism in neurons requires more detailed investigation. Our previous data indicated that both STIMs are involved in Ca^{2+} homeostasis in neurons (Klejman *et al*., 2009), form complexes with endogenous ORAI1 (Gruszczynska-Biegala and Kuznicki, 2013) but play a distinct role in SOCE (Gruszczynska-Biegala *et al*., 2011). In contrast to non-excitable cells, Ca^{2+} influx in neurons is modulated mainly by voltage gated Ca^{2+} channels and ionotropic receptor-operated Ca^{2+} channels. Here we report, that the SOCE inhibitor ML-9 reduces AMPA-induced Ca^{2+} influx by 80%. To assess the role of AMPA receptors (AMPARs) in SOCE, they were inactivated in cortical neurons by their specific inhibitors. As estimated by FURA-2AM single-cell Ca^{2+} measurements

in the presence of CNQX or NBQX, thapsigargin-induced Ca^{2+} influx was decreased 2.2 or 3.7 times, respectively. These results suggest that during SOCE, calcium ions can enter neurons also through AMPA receptors. In addition, we found by co-immunoprecipitation assays, that when Ca^{2+} level is low in the neuronal ER, a physical association of endogenous STIM proteins with endogenous GluA1 or GluA2 subunits of AMPAR occurs. Taken together, these data suggest an involvement of AMPAR in SOCE and its link with STIM proteins.

Supported by funds from a National Science Centre (2011/01/D/NZ3/02051, JGB).

|A15|

The effect of one week of low-threshold stimulation of proprioceptive fibers on glutamatergic and cholinergic innervation of the ankle extensor α-motoneurons on early-phase after complete transection of the spinal cord

Grycz K., Glowacka A., Gajewska-Wozniak O., Ji B., Czarkowska-Bauch J., Skup M.

Nencki Institute of Experimental Biology, Warsaw, Poland
k.grycz@nencki.gov.pl

Seven days of stimulation of low-threshold proprioceptive fibers in the tibial nerve in intact rats leads to synaptic plasticity in the Hoffmann-reflex (H) circuit involving two extensors operating at the ankle joint, i.e., soleus and lateral gastrocnemius (LG) α-motoneurons (MNs). We focused on the effects of stimulation on two types of excitatory inputs to α-motoneurons: the first input is formed by glutamatergic Ia sensory afferents contacting monosynaptically α-MNs; the second one is the cholinergic input originating from V0c – interneurons. We found that one week of continuous burst stimulation of proprioceptive input to LG α-motoneurons is effective in enrichment of their direct glutamatergic but also indirect cholinergic inputs.

Our recent aim is to clarify whether enhancement of signaling to ankle extensor α-MNs, via the same pattern of direct electrical stimulation of tibial nerve, will affect both Ia glutamatergic and cholinergic innervation of α-motoneurons of LG in rats subjected to complete transection of the spinal cord at low-thoracic segments. Tibial nerve was stimulated for 7 days with continuous bursts of three pulses delivered every 25 ms in 4 × 20 minutes sessions daily. Stimulation started on the second day after spinalization. Monitoring of H-reflexes and threshold M-responses recorded from the soleus muscle, allowed controlling strength of stimulation of Ia afferents. LG-MNs were identified with tracer injected intramuscularly. Glutamatergic Ia- and cholinergic C-terminals abutting on LG-MN perikarya were detected by immunofluorescence (IF) using input-specific anti-VGLUT1 and anti-VAChT antibodies, respectively.

The effect of spinalization on frequency of H-reflexes was inconsistent but frequency of complex responses after 2nd and 3rd stimuli in the burst increased after stimulation. Quantitative analysis of confocal images revealed that 7-days after spinalization the number of VGLUT1-IF terminals tended to decrease and stimulation of Ia fibers did not bring change. The number of VAChT-IF terminals did not change either after spinalization or stimulation. The volume of terminals tended to decrease after spinalization. To conclude, one week of continuous burst stimulation of proprioceptive Ia input to LG-MNs, which started early after spinalization, was not sufficient to bring substantial changes in innervation of LG-MNs.

Support: NCN 2013/09/B/NZ4/03306; statutory funds for the Nencki Institute.

|A16|

Treatment with neurotensin-opioid hybrid peptide alleviates inflammation in murine model of contact sensitivity reaction and non-atopic asthma – preliminary results

Kogut E.[1], Kaczynska K.[1], Lipkowski A.[2], Kleczkowska P.[3]

[1]Laboratory of Respiration Physiology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Neuropeptides, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[3]Department of Pharmacodynamics, Centre for Preclinical Research and Technology, Medical University of Warsaw, Warsaw, Poland
ekogut@imdik.pan.pl

Background: Delayed type hypersensitivity (DTH) plays crucial role in pathophysiology of inflammatory disorders like contact sensitivity and non-atopic asthma. Immunization with low molecular compounds and local challenge with the cognate antigen elicit tissue swelling response and asthma like symptoms. The object of the study was

to investigate anti-inflammatory activity of PK20 (hybrid peptide) in experimental murine models of inflammation.

Methods: Non-atopic asthma and contact sensitivity response were induced in mice by skin sensitization with dinitrofluorobenzene (DNFB) followed by intratracheal challenge of dinitrobenzene sulfonic acid (DNS) and topical DNFB application on ears. After hapten challenge and eight hours later, mice were treated intraperitoneally with PK20, neurotensin or endomorphin-2 pharmacophores. In subsequent experiments the effect of neurotensin and opioid receptors blockade on PK20-induced anti-inflammatory activity was examined. Twenty four hours after hapten challenge, bronchoalveolar lavage fluid (BAL) was collected and total amount of inflammatory cells was counted using Burker-Turk chamber. Measurement of ear thickness was performed with engineer's micrometer. Ear swelling was calculated by subtracting swelling recorded for the vehicle-control ear from the swelling recorded for the DNFB challenged ear.

Results: Treatment with PK20 abolished the late phase of ear swelling and significantly reduced the total number of cells in BAL fluid in comparison to group treated with physiological saline. Blockade of neurotensin receptors was more effective in reducing hybrid anti-inflammatory activity than blockade of opioid receptors. These relations were more apparent in the ear thickness measurements than in the bronchoalveolar lavage studies. Application of endomorphin-2 and neurotensin pharmacophores diminished the ear edema and the number of inflammatory cells in BAL, however they were much less potent than PK20 treatment.

Conclusions: Hybrid peptide, PK20 reduces the signs of inflammation: ear swelling and infiltration of inflammatory cells into the lungs. Both neurotensin and opioid pathways seem to be involved in anti-inflammatory activity of tested compound. However, study with application of antagonists indicates stronger contribution of neurotensin component. The mechanism of PK20 action remains unclear and needs further investigation.

The study was supported by a grant from National Science Centre, Poland, no. 2014/13/B/NZ7/02247.

|A17|

The dopaminergic innervation of the orbitofrontal prefrontal cortex (OFC) is significantly altered in the spontaneously hypertensive rats (SHR)

Kolenkiewicz M.[1], Kozlowska A.[2], Rowniak M.[3]

[1]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[2]Department of Human Physiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Department of Comparative Anatomy, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland

malgorzata.kolenkiewicz@uwm.edu.pl

The attention deficit hyperactivity disorder (ADHD) is associated with dysfunctions of the dopamine (DA) system and decreased dopamine activity in the prefrontal cortex (PFC). The orbitofrontal cortex (OFC), which is a part of PFC, may be critical for ADHD. This brain center is involved in response to reward and adjustment animal behavior when the rewarding properties of the reinforcement change. Moreover OFC is important during the developing of addiction and there are several lines of evidence that untreated ADHD is conducive to addiction and abuse. Thus, the question is whether OFC is altered in the individuals affected by ADHD and how the dopamine activity is altered. The aim of this study was to compare DA immunoreactivity in OFC of the spontaneously hypertensive rats (SHR, animal model of ADHD) and Wistar Kyoto rats (WKY, healthy controls).

Frozen brain sections from juvenile (4 weeks old) and adult (10 weeks old), male SHR and WKY rats were processed by single immuonofluorecence using thyrosine hydroxylase (TH; the rate-limiting enzyme of catecholamine biosynthesis) antibody. In the medial orbital cortex (MO), ventral orbital cortex (VO), lateral orbital cortex (LO) and dorsolateral orbital cortex (DLO) immunoreactive structures were carefully counted and the counts were compared between SHR and WKY rats.

The result show that the number of fibers expressing TH in LO, VO and DLO was lower, while in MO higher in the 4 weeks old SHR rats when compared to the WKY strain. In adult animals (10 weeks old) this pattern of TH distribution was reversed. In conclusion, these results suggest that the DA system is significantly altered in the orbitofrontal cortex of the SHR model of ADHD. As OFC mediates cognitive control of behavior, alterations in DA system in this region my lead to diversified symptoms of ADHD such as behaviors that are inappropriate for the context,

premature, poorly planned and often resulting in adverse consequences.

Supported by grant PL-TW II/4/2015.

|A18|

5-HTTLPR polymorphism and serotonin concentration among migraine patients

Kowalska M.[1], Mrzewka B.[1,2], Wieczorek I.[1,2], Kapelusiak-Pielok M.[3], Kozubski W.[3], Dorszewska J.[1]

[1]Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland
[2]Students Scientific Neurobiological Association, Poznan University of Medical Sciences, Poznan, Poland
[3]Chair and Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland

Introduction: Migraine is a primary headache disorder that affects 11% of adults. There are two main, distinct clinically and probably also etiologically, subtypes of the disease: migraine with aura (MA) and migraine without aura (MO). The cortical spreading depression (CSD) is postulated to be involved in pathomechanism of migraine. In the consequence of CSD activation, many vasoactive factors are released, e.g. serotonin (5-HT). The correlation between 5-HTTLPR polymorphism of the serotonin transporter gene and level of 5-HT was found, but the possible impact on migraine remains unclear. It is known that the SS genotype of 5-HTTLPR is associated with lower reuptake of 5-HT, while the SL genotype is linked to impaired function of 5-HT transporter.

Aim of the study: The aim of the study was to analyze 5-HTTLPR polymorphism, 5-HT plasma concentration and clinical features of migraine.

Material and methods: The study included 96 migraine patients (MA: 43, MO: 53; mean age 39 ± 14) and 82 controls (mean age: 38 ± 14). The 5-HTTLPR polymorphism was determined by polymerase chain reaction (PCR) and visualized by electrophoresis. The high performance liquid chromatography with electrochemical detection (HPLC/EC) was used to determine 5-HT plasma level.

Results: Plasma concentration of 5-HT was higher in MA patients than in MO or control group ($p < 0.01$). The SL genotype of 5-HTTLPR polymorphism was more frequent and LL was less frequent in migraine than in controls ($p < 0.01$). The SS genotype was associated with higher 5-HT level only in MA. The correlation between duration of migraine and 5-HT concentration was also observed only in MA: the longer migraine history, the higher 5-HT level. Moreover, it was found, that the level of 5-HT changes with the length of a migraine attack, both in MA and MO.

Conclusion: Serotoninergic system may play more important role in pathogenesis of MA than MO.

|A19|

The changes in density of catecholaminergic fibres in the rostral prefrontal cortex spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY)

Kozlowska A.[1], Kolenkiewicz M.[2], Tsai M.L.[3], Rowniak M.[4]

[1]Department of Human Physiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[2]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Department of Biomechatronic Engineering, National Ilan University, Taiwan
[4]Department of Comparative Anatomy, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland

kozlowska.anna@uwm.edu.pl

Dysfunction of dopamine (DA) neuronal systems leads to several serious emotional disorders and it has been postulated that DA alterations may be key factor in the pathophysiology of attention deficit hyperactivity disorder (ADHD). Dopamine plays a key role in attentional, psychomotor, reinforcing and rewarding behaviors that are deficient in ADHD. The rostral prefrontal cortex (rPFC) is a part of dopamine neuronal systems and it has several features which may be critical for ADHD development. One of them is the fact that rPFC has been directly associated with attentional processes, visceromotor activity, decision making and goal directed behaviors. The second fact is that decreased DA release in rPFC impairs the actions of this area. The aim of this study was to compare DA immunoreactivity in the rostral prefrontal cortex of the spontaneously hypertensive rats (SHR, animal model of ADHD) and Wistar Kyoto rats (WKY, healthy controls). Juvenile (4 weeks old) and adult (10 weeks old), male SHR and WKY rats were used in the study. Frozen 10 µm-thick brain sections comprised rPFC were stained by standard single immunofluorecence using thyrosine hydroxylase (TH; the rate-limiting enzyme of catecholamine biosynthesis) antibody. Following parts of rPFC were analyzed: infralibic (IF), prelimbic (PrL) and cingulate cortex (Cg). In all these

areas immunoreactive structures were counted manually using test frames.

The result show that the densities of fibers containing TH in 4 weeks old SHR rats were lower in all analyzed areas of the rPFC in comparison with WKY strain. Moreover, in 10 weeks old animals the same differences were still observed in the infralibic cortex, while in the prelimbic and cingulated cortices they have been erased.

In conclusion, these results suggest that DA system in the rostral prefrontal cortex of the SHR model of ADHD is hypofunctional. As dopamine and rPFC play key roles in attentional processes, lowered DA expression in this brain region fits well with inattention observed in ADHD affected individuals.

Supported by grant PL-TW II/4/2015 and MOST 104-2923-B-197-001-MY3.

|A20|

TRP channels are engaged in memory consolidation and reconsolidation in passive avoidance task in one-day old chicks

Krolik A., Stafiej A., Slomka M., Salinska E.

Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

andy.krolik@gmail.com

The stimulation of both ionotropic and metabotropic glutamate receptors and influx of calcium ions (Ca^{2+}) into neurons is a crucial step in intracellular cascade of memory formation. Recently the existence of additional mechanism involved in intracellular Ca^{2+} increase, triggered by internal signals like increase of Ca^{2+} within the cell and activation of G protein coupled receptors, was demonstrated. This mechanism involves transient receptor potential (TRP) channels. The aim of our study was to investigate the participation of TRP channels in intracellular mechanisms engaged in memory consolidation and reconsolidation.

The model of passive avoidance task in one day old chicks was used. Chicks were injected with non-specific TRP channels antagonist SKF96365 or with three different concentrations of 2-APB, the inhibitor of IP3 receptors, which in small concentrations (~10 μM) inhibits also TRP channels. The injections were made at different times before and after training, to find the most effective time. The injection of each antagonist immediately after training resulted in task amnesia when tested 24 h later. The injection of SKF96365 immediately after training resulted in constant amnesia that manifested 1.5 h after training, whereas amnesia after injection of 2-APB was observed as early as 30 min after training. The effect of application of TRP channels antagonist SKF96365 and 10 μM 2-APB on memorizing of the task in comparison with the effects of antagonists of mGluR1 and mGluR5, the receptors that trigger IP3 release, showed similarities when memory was tested 2 h and 24 h training. Application of SKF96365 or 10 μM 2-APB immediately after reminder given 2 h after initial training, resulted in transient amnesia of the task that manifested in a short time after reminder and lasted for as long as 6 h. Our results show that inhibition of both TRP channels and IP3 receptors has a strong impact on both memory formation and reconsolidation.

Studies supported by National Science Centre grant G1136-2014/15/B/NZ4/04487.

|A21|

Overexpression of parkin protects PC12 cells against alpha-synuclein evoked mitochondria damage and cell death

Lenkiewicz A.M., Wilkaniec A., Adamczyk A.

Department of Cellular Signalling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Parkin, an ubiquitin E3 ligase, that is responsible for the clearance of damaged proteins, is linked to rare familial forms of Parkinson's disease (PD) through loss-of-function mutations and to sporadic PD through posttranslational inactivation. However, the detailed mechanism underlying the neuroprotective function of parkin in dopaminergic neurons, especially its role in alpha-synuclein (ASN) evoked toxicity, is still unclear. Recent studies have focused on parkin's role in mitochondrial biogenesis and turnover, including mitochondrial fission/fusion as well as mitophagy. The aim of this study was to investigate the protective role of parkin in ASN-induced mitochondrial damage. Investigations were performed on PC12 control cells and PC12 cells with parkin overexpression treated with ASN oligomers as well as in cells with parkin knock-down. We demonstrated that exogenous ASN induced overproduction of free radicals including nitric oxide (NO), resulting in parkin S-nitrosylation and alteration of its activity. Concomitantly, in ASN treated cells the parkin protein level was

decreased, while no significant difference in mRNA level was found. Additionally, both NO stress, induced by ASN oligomers, as well as parkin knock-down triggered mitochondrial dysfunction, followed by significant decrease in mitochondrial membrane potential, overproduction of mitochondrial superoxide anion and depletion of cellular ATP level. Moreover, changes in the expression of proteins that regulate mitochondria biogenesis (peroxisome proliferator-activated receptor γ co-activator-1α, PGC-1α), fission (dynamin-related protein Drp1) and fusion processes (Opa1, Mitofusin-2, Mfn2) were observed. These events create a death-prone milieu that contributes to the loss of dopaminergic cells. Finally, we showed that parkin overexpression prevented mitochondrial superoxide production and attenuated ASN-evoked PC12 cell death, pointing to the importance of parkin in ASN-mediated toxicity. These findings may thus provide a molecular link between parkin dysfunction and ASN induced mitochondrial impairment in sporadic PD. We suggest that amelioration of parkin's function may be a novel therapeutic target to treat PD.

Supported by NSC grants: 2012/05/B/NZ3/02047 (to A.A.) and 2013/09/D/NZ3/01359 (to A.W.).

|A22|

Protein kinase B signaling pathway in glioblastoma cells transfected with the liver-type glutaminase

Majewska E.[1], Szeliga M.[1], Marquez J.[2], Albrecht J.[1]

[1]Department of Neurotoxicology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Molecular Biology and Biochemistry, Faculty of Sciences, Campus de Teatinos, University of Málaga, Málaga, Spain

Glutamine (Gln) plays a crucial role in the metabolism of tumors of different origin, including gliomas. Deregulated expression/activity of glutaminase (GA, EC 3.5.1.2), an enzyme converting Gln to glutamate (Glu) and ammonia is a characteristic feature of many cancer cell lines and tumors. There are two coding genes for GA: GLS and GLS2. Mounting evidence suggests that proteins encoded by either of the genes play opposing role in tumorigenesis. In glioblastoma (GBM), the most aggressive brain tumor, GLS encoding kidney-type isoforms (KGA and GAC) is highly expressed, while expression of GLS2 coding for liver-type isoforms (LGA and GAB) is hardly detectable. Previous studies revealed that transfection of human glioblastoma T98G cell line with a sequence encoding GAB suppressed malignant phenotype of these cells and altered expression level of 85 genes (Szeliga *et al*., 2009). The cells transfected with GAB (herein referred to as TGAB) are more sensitive to alkylating agents used in GBM therapy (Szeliga *et al*., 2012) and to oxidative stress (Martin-Rufian *et al*., 2014). Activation of the PI3K/AKT pathway has been documented in GBM, but it is also observed in different cell types upon oxidative stress. Here we tested the hypothesis, that transfection with GAB modulates the PI3K/AKT pathway. Western blot analysis revealed a ~45% decrease in the level of phosphorylated AKT in TGAB cells as compared T98G cells and TpcDNA (T98G transfected with an empty vector). The level of the unphosphorylated AKT remained unchanged. No differences between TGAB and T98G or TpcDNA cells were observed in the level of PTEN, a negative regulator of AKT signaling pathway. In conclusion, our results suggest that transfection with GAB modulates AKT signaling pathway in T98G cells. A more detailed analysis of this phenomenon is currently conducted in our laboratory.

Supported by: the National Scientific Leading Centre (KNOW-MMRC) project (to EM) (KNOW1).

|A23|

Circulating levels of miR-1, miR-29 and miR-30 are associated with BDNF level in serum of patients with Parkinson disease

Malczynska P.[1,3], Brodacki B.[4], Langfort J.[2], Chalimoniuk M.[1]

[1]Department of Cellular Signaling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Pharmacology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[3]Faculty of Agricalture and Biology, Warsaw University of Life Sciences, Warsaw, Poland
[4]Department of Neurology, Military Medical Institute, Warsaw, Poland

Numerous studies have shown that brain-derived neurotrophic factor (BDNF) regulates number of functions in the nigrostriatal system. It demonstates a neuroprotective effect against degeneration of dopaminergic neurons and improves both memory and motor activity. Reduced CNS concentration of BDNF in the elderly is accompanied with increased number of incidents of neurodegenerative diseases. Small non-coding RNAs of 21-24 nucleotides long

(miR-1, miR-29, miR-30-5a) play an essential role in the regulation of BDNF gene expression. The aim of the present study was to determine the concentration of BDNF and miR-1, miR-29, miR-30-5a in the serum of patients with idiopathic parkinsonism (iPD). The concentration of BDNF was determined by ELISA (R&D System, Minneapolis, MN, USA). Selected miRNAs were determined by array cart and real-time qPCR using specific primers TaqMan miRNA assay (Life Technology, Carlsbad, CA, USA) in the serum of patients with iPD and age-matched healthy subjects. The serum concentration of BDNF decreases with aging in healthy subjects. Moreover, the concentrations of BDNF, miR-1, miR-29 and miR-30 were statistically lower in the serum of patients with iPD compared with age matched healthy subjects. These results revealed that decreasing of miRNA-s levels was associated with reduction of BDNF level in serum of iPD patients. Our study provides a preliminary evidences that the regulation of BDNF level by miRNA may play a role in preventing of the neurodegenerative processes in nigrostriatal system.

|A24|

Role of nitric oxide synthase (NOS) in generation of oxidative/nitrosative stress in the cerebral cortex of rat with thioacetamide induced acute liver failure (ALF)

Milewski K., Fresko I., Albrecht J., Zielinska M.

Department of Neurotoxicology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
kmilewski@imdik.pan.pl

Acute liver failure (ALF) is associated with deregulation of NMDA/cGMP/NO signaling and oxidative/nitrosative stress in the brain. However, the relative roles of the different NOS isoforms and the mechanisms underlying alterations in their activities during ALF are not fully clear. Here we investigated gene and protein expression of NOS isoforms, NOS activity, eNOS uncoupling and total NO production in cerebral cortex of rats with thioacetamide (TAA)-induced ALF. Sprague Dawley rats (250-280 g) received three i.p. injections of TAA (300 mg/kg) at 24 h intervals. The brain cortex expression of NOS isoforms (eNOS/iNOS/nNOS) was measured by Real-time PCR and Western Blot, NOS activity was tested by monitoring the conversion of radiolabeled arginine to citrulline. Reactive oxygen species (ROS) were quantified in the presence of NOS substrate L-arginine, using the carboxy-H2 DCFDA probe. NO was measured with the Griess procedure. The eNOS expression was decreased, whereas the eNOS dimmer/monomer ratio and nNOS/iNOS expression were elevated in TAA treated rats. While the total NOS activity was decreased, the iNOS activity was elevated and NO concentration tended to increase. ROS production was elevated by TAA. Unspecific NOS inhibitors L-NAME and LNNA attenuated ROS production in both control and TAA rats, but with higher efficiency in the latter case. Ca^{2+} chelation had almost the same effect as pharmacological NOS inhibition suggesting that Ca^{2+}-independent iNOS activity is not the main source of ROS. Incubation with high dose of tetrahydrobiopterin, which is critical for eNOS dimerization and subsequent NO production, also reduced ROS production indicating the eNOS uncoupling phenomenon in TAA cortex. The study points to eNOS downregulation due to lowered protein expression and uncoupling as a mechanism contributing to enhanced superoxide anion formation, and confirms the role of iNOS/nNOS in enhancing NO synthesis in ALF-affected brain.

|A25|

Toxicological research of 1-methyl-1,2,3,4-tetrahydroisoquinoline an exo/endogenous amine with antidepressant-like activity – *in vivo*, *in vitro* and *in silico* studies

Mozdzen E.[1], Babinska I.[2], Wojcikowski J.[3], Antkiewicz-Michaluk L.[1]

[1]Department of Neurochemistry, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland
[2]Department of Pathophysiology, Forensic and Administration of Veterinary Medicine, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Department of Pharmacokinetics and Drug Metabolism, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Tetrahydroisoquinolines, the most numerous naturally occurring alkaloids, include 1-methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ) which demonstrates significant neuroprotective activity observed in various neurotoxicity models and has structural similarity to dopamine (DA). It can interact with agonistic conformation of DA receptors. 1MeTIQ inhibits the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) as well as production of free radicals and shifts DA catabolism toward the catechol-O-methyltransferase (COMT)-dependent O-methylation, and such mechanism of action seems to be important for its neu-

roprotective activity. It has been found that 1MeTIQ inhibits both monoamine oxidase A (MAO-A) and B (MAO-B) enzymes activity and increases neurotransmitters level in the brain. That is more, 1MeTIQ shows significant antidepressant-like effect in the FST and the reserpine model of depression in rats. Therefore, this compound might be effective for the depression therapy in a clinical setting but the success of this drug is determined not only by its good efficacy but also by an acceptable ADMET profile. ADMET prediction use in combination with in vivo and in vitro studies greatly simplifies the search for new, safer and effectively acting drugs. The aim of this study was to investigate the degree of histopathological changes in different rat tissues (liver, kidney, lung) after acute and chronic administration of 1MeTIQ. Additionally, prediction of its properties in terms of absorption, distribution, metabolism, elimination and toxicity in the human body was performed. The obtained data did not show extensive and significant toxic effects of tested substance in in vivo and in vitro studies in rats, and in silico ADMET prediction in the human body. These results can help to discover or model a new effective and safe antidepressant substance and have important significance in the treatment of depression in clinic. Additionally, the use in the treatment of depression substance existing endogenously, having neuroprotective, antioxidant and antidepressant-like effects in the central nervous system (CNS) might also be beneficial in controlling the adverse CNS inflammatory processes often accompanying depression.

Acknowledgements: Grant No. 2013/11/N/NZ7/00358.

|A26|

Hyperbaric oxygen preconditioning-induced alterations in the expression of proteins, associated with progenitor cells and apoptosis, are modified by a proteasome inhibitor in the ischemic brain

Ostrowski R.P., Stepien K., Pucko E., Wojda R., Matyja E.

Department of Experimental and Clinical Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Nestin is a marker of central nervous system progenitor cells, pointing to the potential for cell repair and tissue remodeling after brain injuries. The p53, although have been named the guardian of the genome, may trigger apoptosis of injured cells after global brain ischemia, unfavorably for investigational therapeutic interventions.

Studies have found that loss of p53 may facilitate nestin expression in cerebral tissues. We evaluated the expression of nestin and p53 in the rat brain in a model of global cerebral ischemia induced by the occlusion of carotid arteries (two-vessel occlusion; 2VO) associated with hypotension. We hypothesized that hyperbaric oxygen preconditioning (HBO-PC) will reduce p53 expression in the ischemic brain in a proteasome-dependent fashion and will enhance the expression of nestin under these conditions. We also performed Klüver-Barrera stain, synaptophysin and NeuN immunohistochemistry on rat brain sections to evaluate neuronal, synaptic and myelin injuries after global ischemic insult and the effect of HBO-PC.

The adult Wistar rats were allocated into following groups: sham operation, 5 minute 2VO untreated or preconditioned with HBO-PC (2.5 ATA for 1 hour for 5 consecutive days) before ischemia as well as preconditioned in combination with proteasome inhibitor MG132 prior to each HBO-PC session. The brains were collected at days 1 and 7 for histological analysis.

At 7 days, the majority of nestin positive cells was found in the hippocampus and periventricularly in the brain, predominantly within HBO-PC group.

Synaptophysin immunoreactivity decreased at one week following global cerebral ischemia. With preconditioning, the sparing of synaptophysin positive structures in CA1 and cerebral cortex was observed 7 days after reperfusion. The level of p53 was elevated in the CA1 and cerebral cortex on day 1 after ischemia, while in surviving neurons after 7 days. Reduction of p53 expression was observed with the preconditioning. Klüver-Barrera and NeuN stains showed damage of myelin, CA1 and cortical neurons, reduced in preconditioned group. However, MG132 combined with HBO-PC abolished beneficial changes in the postischemic brain. Thus proteasome system may be involved in the mechanism of HBO-PC-induced reduction in the p53 levels and tissue repair after brain ischemia.

|A27|

Possible influence of kinin B1 receptor on the blood-brain barrier integrity during autoimmune encephalomyelitis in rats

Podsiadlo K., Dabrowska-Bouta B., Grygorowicz T., Sulkowski G., Struzynska L.

Laboratory of Pathoneurochemistry, Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

According to the previous studies, mammalian central nervous system presents all components of the kallikrein-kinin system. Kinins are vasoactive and pro-inflammatory peptides whose biological effects are mediated by two G-protein-coupled receptors: B1 and B2. As suggested, activation of B1R leads to the induction of inflammation by the release of pro-inflammatory cytokines and increased vascular permeability. Since inflammation and the blood-brain barrier (BBB) disruption are main components of multiple sclerosis (MS), there are reasons to investigate the role of B1 receptor in these processes in the animal model of the disease, which is experimental autoimmune encephalomyelitis (EAE). Female Lewis rats were immunized by injection of inoculum containing homogenate of guinea pig spinal cord. Animals were monitored daily for clinical signs and loss of weight and sacrificed in different stages of the disease. The second group was administered with DALBK (B1R antagonist) after immunization. The expression of B1R was analyzed by W-B; gene expression was quantified by RT-PCR. The level of cytokines was assessed using RayBio Rat Cytokine Antibody Array. Immunohistochemical studies were also performed on isolated fraction of microvessels towards protein markers of BBB tightness.

We noticed the increased level of B1R protein in the rat brain during the symptomatic phase of EAE. Administration of DALBK significantly improved the condition of animals by reducing the intensity of neurological symptoms and delaying the onset of the disease. Using a confocal microscope, we observed lowered immunoreactivity of pericytes receptor PDGFβ and tight junctions proteins (ZO-1, claudin 5) in microvessels' fraction obtained from EAE rats which increased after DALBK administration. Also preliminary analysis showed increased protein level of cytokines: IFN-γ, IL-1β, IL-6, TNF-α, VEGF in EAE animals, which tends to decrease after DALBK treatment in symptomatic phase of the disease. Results show that B1R-mediated proinflammatory effect of kinins may be involved in pathomechanisms operating during the pre-onset phase of EAE resulting in disturbances in BBB integrity through the influence on tight junctions proteins.

This work was made under MMRC-KNOW 2013-2017 project (KNOW-13).

|A28|

The catecholaminergic system of the dorsolateral prefrontal cortex is down regulated in the juvenile spontaneously hypertensive rats (SHR)

Rowniak M.[1], Kolenkiewicz M.[2], Kozlowska A.[3]

[1]Department of Comparative Anatomy, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Poland
[2]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Department of Human Physiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland

mrowniak@uwm.edu.pl

The motor cortex (MC) is the dorsolateral region of the prefrontal cortex (PFC) involved in planning, control, and execution of voluntary movements. More recent findings also suggest that MC plays role in learning and cognition. The rat MC, like in other mammals, is commonly divided into primary (M1) and secondary (M2) motor cortices. Both M1 and M2 are involved in the execution of voluntary movements via their direct and parallel projections to the spinal cord. The MC as other parts of PFC is strongly innervated by dopaminergic fibers and dopamine (DA) and is essential for proper motor control. Any abnormalities in DA neurotransmission may lead to serious disorders affecting motor and/or emotional behaviors. The attention deficit hyperactivity disorder (ADHD) is characterized by abnormalities in motor and emotional behaviors and recent reports indicate dysfunction in DA neurotransmission may be one of the key factors in the pathophysiology of disease. The question is whether MC is engaged in ADHD and whether DA neurotransmission is altered in MC in affected individuals.

The aim of this study was to track changes in DA immunoreactivity in the motor cortex of the spontaneously hypertensive rats (SHR) – animal model of ADHD. Frozen brain sections from juvenile (4 weeks old) and adult (10 weeks old) male SHR and WKY (Wistar Kyoto) rats were processed by single immuonofluorecence using thyrosine hydroxylase (TH; the rate-limiting enzyme of catecholamine biosynthesis) antibody. In M1 and M2 immunoreactive structures were

counted manually and compared between SHR and WKY rats. The result show that the number of fibers exhibiting immunoreactivity for TH in both M1 and M2 was lower in the 4 weeks old SHR rats when compared to the WKY strain. Furthermore, in 10 weeks old SHR and WKY rats the number of TH expressing fibers was similar in the M2 while in the M1 this number was still significantly lower in SHR rats.

In conclusion, present results indicate that dopaminergic inhibition of the motor cortices is significantly lowered in ADHD affected individuals what may be one of the reasons of the motor hyperactivity observed in this syndrome.

Supported by grant PL-TW II/4/2015.

|A29|

Neuroprotection of raloxifene and bazedoxifene against hypoxia depends on developmental stage but not on caspase-3 related apoptosis

Rzemieniec J.[1], Wnuk A.[1], Litwa E.[2], Lason W.[1], Kajta M.[1]

[1]Department of Experimental Neuroendocrinology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland
[2]Behavioural Pharmacology Laboratory, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Hypoxia occurs under different circumstances, such as stroke, obstructive sleep apnea, mountain sickness or cancer. The brain of newborn mammals has been considered less sensitive to oxygen supply than the adult one, but resistance of the immature brain to hypoxia has its limitations. Selective estrogen receptor modulators (SERMs) represent an alternative to estrogen devoid of its side-effects and acting as estrogen receptor agonists or antagonists in a tissue-specific manner. SERM representatives raloxifene and recently approved bazedoxifene are used in clinical practice against osteoporosis but their neuroprotective properties are only partially recognized. Our previous study has shown that raloxifene exerts neuroprotection against hypoxia-induced damage in mouse hippocampal cell cultures. However, the roles of apoptosis and the stages of neuronal development in neuroprotective capacity of SERMs have not been clarified. Furthermore, knowledge about neuroprotective potential of bazedoxifene is limited.

Therefore, the aim of the present study was to investigate neuroprotective potential of raloxifene and bazedoxifene in mouse neocortical cells at different stages of neuronal development with special concern on apoptosis-dependent effects.

Our experiments were performed on mouse primary neocortical cell cultures. On 2, 7 and 12 day *in vitro* (DIV) the cells were treated with raloxifene (0.01-10 μM) or bazedoxifene (0.01-5 μM) and subjected to 18 h hypoxia [5% CO_2/95% N_2]. Caspase-3 activity and lactate dehydrogenase (LDH) release were measured after 6 h of reoxygenation.

We have shown that 18 hours of hypoxia increased LDH release by 25, 91 and 61% at 2, 7 and 12 DIV, respectively. Hypoxia caused also about 35% enhancement of caspase-3 activity, but only at 7 and 12 DIV. Raloxifene and bazedoxifene (0.01-1 μM) inhibited the hypoxia-induced LDH release in all *in vitro* stages of neuronal development. Raloxifene and bazedoxifene did not change the caspase-3 activity but at concentrations higher than 1 μM evoked neurotoxic effect.

These data demonstrated strong neuroprotective capacity of raloxifene and bazedoxifene that revealed particularly at later developmental stages and did not involve caspase-3-dependent apoptosis. Our study may be utilized in searching for new SERM-based tools to protect developing brain against hypoxia.

Joanna Rzemieniec and Agnieszka Wnuk are holders of scholarship from the KNOW sponsored by Ministry of Science and Higher Education, Republic of Poland.

The study was supported by statutory funds of the Institute of Pharmacology, Polish Academy of Sciences.

|A30|

Exposure of cultured mouse astrocytes to NMDA inhibits expression of mRNAs coding for astroglia-specific proteins by a calcium-dependent mechanism

Skowronska K., Obara-Michlewska M., Czarnecka A., Zielinska M., Albrecht J.

Departament of Neurotoxicology, Mossakowski Medical Research Centre, Polish Academy of Science, Warsaw, Poland

NMDA receptors are present in rodent astrocytes but their physiological role beyond generation of intracellular calcium signals has not been studied in much detail. This laboratory has previously shown that prolonged treatment of cultured rat astrocytes with glutamate or NMDA decreases the expression of the astroglia- specific inward rectifying potassium channel, Kir 4.1 (Obara-Michlewska *et al*., Neurochem Int, 2015). Here, expression of mRNAs

coding for Kir4.1, the water channel aquaporin-4 (AQP4) and glutamine synthetase (GS) in primary cultures of mouse cortical astrocytes exposed for 8-72 h to NMDA was analyzed using real-time PCR. The effect of NMDA on Kir4.1 was shown to be biphasic: a decrease after 8 h exposure was followed by an increase at 72 h. Expression of AQP4- and GS mRNA was found decreased at both 8 h and 72 h of incubation. The results showed that the NMDA-induced changes were abolished in cultures in which expression of the NR1 subunit of the NMDA receptor was blocked with NR1 siRNA. For all the three mRNAs, the decrease of expression at 8 h was observed when incubations were carried out in the presence, but not in the absence of Ca^{2+} ions in the medium, suggesting that the effects of NMDA receptor stimulation were ionotropic in nature. The ionotropic mechanism of astrocytic NMDA receptors function is also supported by observation that NMDA-induced Kir4.1 mRNA decrease is not prevented by ryanodine, the inhibitor of calcium release from sarcoplasmic reticulum.

The effects of NMDA occurred by a mechanism bypassing changes in subunit composition of the NMDA receptor, which appeared unchanged at the mRNA level. The NMDA-induced decrease of GS mRNA was accompanied by marked decrease of GS activity at 8 h. The results suggest that durable activation of astrocytic NMDA receptors may be relevant to astrocytic dyshomeostasis in neurological disorders associated with excessive glutamatergic neurotransmission.

Supported by NCN grant 2013/08/M/NZ3/00869 to JA.

|A31|

Sequence analysis and structural modelling of the litaf/simple protein involved in the CMT1a disease

Sokolowska B.[1], Sinkiewicz-Darol E.[2], Kabzinska D.[3], Czerwoniec A.[4], Lesyng B.[1], Kochanski A.[3]

[1]Bioinformatics Laboratory and [3]Neuromuscular Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Provincial Polyclinical Hospital of L. Rydygiera, Torun, Poland
[4]Bioinformatics Laboratory, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland

Charcot-Marie-Tooth (CMT) disorders represent a heterogeneous group of diseases of the peripheral nervous system (various CMT forms) with a prevalence of 1 : 2500. CMT is characterized by a slowly progressive atrophy of distal muscles associated with distal sensory disturbances. Mutations in approximately 100 genes have been identified in peripheral neuropathies. This study is focused on patients with the CMT1A form. No biomarkers of CMT1A were identified, however, the Ile92Val (c.274A>G) sequence variant in the LITAF/SIMPLE gene correlates with an earlier age of onset of CMT1A [Neurogenetics 2015; 16: 27-32]. Analysis of sequences of the LITAF/SIMPLE gene in CMT1A patients, followed by a structural modeling of the LITAF/SIMPLE protein, were carried out. Secondary structure analysis, 3D-order analysis, identification of protein-RNA interaction sites as well as tertiary fold-recognition (FR) were carried out using the GeneSilico metaserver gateway [http://genesilico.pl/meta2/]. Also the MOE modelling environment was used. Significance of the Ile92Val substitution was analyzed in the proposed approach – it may affect the clinical course of the disease.

Summarizing, the present results show that: (i) molecular diagnostics of CMT1A confirms the clinical diagnosis, but its value is limited by current clinical technologies and procedures, (ii) the I92V LITAF sequence variant indicates that patients may be predispose to an earlier age of onset of the CMT1A disease, in particular, (iii) detection of the c.274A>G substitution in the LITAF gene provides an important prognostic information. Finally, the Ile92Val polymorphism is proposed as the genetic marker of the CMT1A disease.

The study was supported by statutory budget of the MMRC PAS, computations and analysis were carried out using the computational infrastructure of the Biocentrum-Ochota project.

|A32|

Assessment of the therapeutic potential of hyperbaric oxygen combined with selected isothiourea derivative (ZKK-3) in malignant glioma treatment *in vitro*

Stepien K., Ostrowski R.P., Pucko E.B., Zielinska M., Matyja E.

Department of Experimental and Clinical Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Objectives: Treatment of patients with high grade gliomas remains ineffective. It is considered to be related with hypoxia of neoplastic tissue. It can be assumed

that improvement of tissue oxygenation, achieved using high-pressured pure oxygen, can enhance the therapeutic potential of cytotoxic compounds. The aim of this study was to investigate if hyperbaric oxygen (HBO) administration will be beneficial for the previously documented anti-tumour properties of novel isothiourea derivative as well as oxygenation status of glioblastoma cells *in vitro*.

Methodology: Human glioblastoma T98G cell line (WHO grade IV) was cultured in medium supplemented with N,N'-dimethyl-S-(2,3,4,5,6-pentabromobenzyl)-isothiouronium bromide (ZKK-3). Cells were exposed to various oxygen conditions: normoxia, hypoxia, HBO, double hypoxia or hypoxia followed by HBO. It was investigated how HBO administration influence the proliferation and viability of glioma cells in comparison to normoxic and hypoxic conditions. Impact of HBO on the expression of HIF-1α protein was also examined. The proliferation of glioma cells was assessed 24 hours after ZKK-3 treatment using Multisizer 3 Beckman Coulter. The viability of T98G cell line was evaluated 24 and 48 hours post ZKK-3 administration by CellTiter 96®AQueous One Solution Cell Proliferation Assay (Promega). HIF-1α level in cell lysates was determined with ELISA test (HIF-1A ELISA Kit, Thermo Scientific).

Results: Administration of hyperbaric oxygen enhanced anti-proliferative properties of tested compound. Also the viability of neoplastic cells significantly decreased under the influence of ZKK-3/HBO treatment compared to normoxic conditions. Differences were more pronounced and observed for lower ZKK-3 concentrations when HBO administration was compared with hypoxia groups. Under low oxygen conditions expression of HIF-1α was markedly increased. On the other hand, exposure to HBO did not change protein content in cell lysates in relation to normoxia. Moreover, HBO was able to reduce HIF-1α level previously elevated as the result of existing hypoxia.

Conclusions: Hyperbaric oxygenation improves antitumour properties of selected pentabromobenzylisothiourea against malignant glioma cells *in vitro*. It is probably connected with reduction of hypoxia state which may be indicated by diminution of HIF-1α protein expression after HBO application.

Acknowledgement: The research was supported by the KNOW-MMRC project and Foundation for the Development of Diagnostic and Therapy.

|A33|

Modulation of the oxidative stress in EAE rat brain by glutamate receptors antagonists

Sulkowski G., Dabrowska-Bouta B., Struzynska L.

Laboratory of Pathoneurochemistry, Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Experimental autoimmune encephalomyelitis (EAE) is the main animal model for the investigation of pathomechanisms of multiple sclerosis (MS). So far the etiology of MS is unknown. Neurodegeneration in MS/EAE pathology is initiated by microglia activation and mediated by oxidative stress, excitotoxicity and inflammation. The elevation of glutamate in cerebro-spinal fluid, as well as changes in the expression of glutamate receptors (GluRs) and excitatory amino acids transporters (EAATs) were observed in brains of MS patients. In the present studies we tested whether NMDA glutamatergic receptor antagonists, amantadine and memantine, influence parameters of oxidative stress in different phases of EAE. Markers of oxidative stress such as lipid peroxidation (expressed by the level of malondialdehyde – the final product of polyunsaturated fatty acids peroxidation in cell membranes), the level of sulfhydryl groups (–SH), and expression of antioxidant enzymes were examined in the CNS. We noticed statistically significant reduction of both protein- and non-protein –SH groups level by about 40% and 20%, respectively. We also observed the increase in the level of lipid peroxidation by about 35% and changes in the expression of different forms of superoxide dismutase (SODs) during the course of EAE. Administration of NMDAR antagonists (amantadine and memantine) to EAE rats significantly improved analyzed parameters. We noted increase of both protein- and non-protein –SH groups level and decrease in the level of lipid peroxidation. Treatment with NMDAR antagonists changed also the expression of antioxidative enzymes – SODs. Obtained results indicate that the significant amount of ROS generated during EAE results from NMDAR-mediated processes. NMDAR antagonists (amantadine and memantine) are efficient to counter reactive oxygen species (ROS) in rat brains during EAE pathology being effective as antioxidants.

|A34|

Impact of triclosan on expression of NMDA receptor subunits in mouse neocortical neurons

Szychowski K.A.[1,2], Wnuk A.[3], Binduga U.[1], Kajta M.[3], Wojtowicz A.K.[2]

[1]Department of Public Health, Dietetics and Lifestyle Disorders, Faculty of Medicine, University of Information Technology and Management, Rzeszow, Poland
[2]Department of Animal Biotechnology, Agricultural University in Cracow, Cracow, Poland
[3]Department of Experimental Neuroendocrinology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Triclosan (TCS) is an antimicrobial agent used extensively in personal care and sanitizing products such as soaps, toothpastes, and hair products. TCS has been incorporated into growing number of medical products as well as in household items such as plastic cutting boards, sport equipment, textiles and furniture. A number of studies has shown presence of TCS in different human tissues such as blood, adipose tissue, liver, brain and in breast milk or urine. N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels widely expressed in the central nervous system and play key roles in excitatory synaptic transmission. NMDARs are heteromeric complexes incorporating different subunits within a repertoire of three subtypes: GluR1, GluR2 and GluR3. There are four different GluR2 subunits (A, B, C and D) of which the most important in neuroplasticity and excitotoxicity are GluN2A and GluN2B.

The aim of the present study was to investigate the impact of TCS on the expression of GluN1, GluN2A and GluN2B NMDA receptor subunits in *in vitro* cultured mouse neocortical neurons.

The cultures of the neocortical neurons were prepared from Swiss mouse embryos on 15/16 day of gestation. The cells were cultured in phenol red-free Neurobasal medium with B27 and glutamine. After 7 days of culture *in vitro*, neurons were exposed to 10 μM of TCS. After 3 and 6 h, mRNA expression of GluN1, GluN2A and GluN2B NMDA receptor subunits was studied. Additionally, protein expression of NMDARs was measured.

Our preliminary data demonstrated that in the presence of 10 μM of TCS, after 3 h mRNA expression of GluN1, and GluN2A NMDA receptor subunits decreased. Similar pattern was observed in respect to protein expression of the receptor subunits after 3 h treatment with TCS. After 6 h exposure to TCS mRNA expression of GluN1 subunit continued to decrease, but mRNA expression of GluN2B significantly increased, possibly because of secondary effects of TCS.

In summary, the presented study demonstrated that GluN1, GluN2A and GluN2B NMDA receptor subunits are involved in TCS mechanism of action. Since TCS interacts with NMDA receptor subunits, it can also affect the proper functioning and development of the nervous system.

Supported by NCN grant 2014/13/N/NZ4/04809.

|A35|

The impact of tianeptine on the inflammatory status of brain in prenatally stressed rats

Trojan E., Slusarczyk J., Glombik K., Chamera K., Basta-Kaim A.

Department of Experimental Neuroendocrinology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland
trojan@if-pan.krakow.pl

Introduction: There is a robust evidence that dysregulation of immune system may be related to the pathophysiology of depressive disorder. The disturbances in the expression of pro-inflammatory cytokines in central nervous system are of particular interest. It has been shown that alterations in the environment during prenatal period, substantial for brain development, leads to long-lasting adverse effects. In the last years, stressful events during pregnancy have received an increased attention, because they influence brain homeostasis especially by affecting the brain immune system. The aim of this study was to examine the impact of prenatal stress procedure on the behavioral and biochemical changes in the IL-1b, IL-18 and TGF-b level in the frontal cortex and hippocampus of adult offspring rats. Moreover the impact of chronic treatment of an antidepressant tianeptine on the above mentioned parameters were tested.

Material and methods: Pregnant rats were subjected to stress sessions from 14th day of pregnancy until the delivery. At 3 months of age, control and prenatally stressed rats were tested for behavioral changes in forced swimming test. After behavioral verification, rats were chronically treated with tianeptine. Two weeks later the animals' behavior was tested again and levels of IL-1b, IL-18 and TGF-b in hippocampi and frontal cortices were determined by ELISA test.

Results: The obtained data showed that prenatal stress causes in adult offspring depression-like behavior. An increase in immobility and a decrease in swimming and

climbing behavior in the forced swim test were observed. Furthermore, the evaluation of the protein level showed increase in the IL-1b, IL-18 level and decrease in the TGF-b level in frontal cortices and hippocampi in adult rats offspring after prenatal stress procedure. Interestingly the chronic treatment of tianeptine not only normalized the behavioral disturbances but also the protein level of analyzed cytokines in both investigated brain structures.

Conclusions: Our study clearly demonstrated that prenatal stress procedure leads to persistent behavioral and biochemical disturbances in adult offspring rats. It also appears that the cytokines' systems can be an attractive target for antidepressant drug action.

This research was supported by the grant no. 2013/09/B/NZ7/04096, National Science Centre, Poland.

|A36|

Activation of the nucleus accumbens with nicotine and caffeine in the rat – results of immunocytochemical studies

Waskow M.[1], Lietzau G.[2], Steliga A.[1], Wojcik S.[2], Morys J.[2], Kowianski P.[1,2]

[1]Department of Health Sciences, Pomeranian University in Slupsk, Slupsk, Poland
[2]Department of Anatomy and Neurobiology, Medical University of Gdansk, Gdansk, Poland

Nicotine and caffeine belong to the most frequently consumed psychostimulating substances all over the word. Their action is concerned with activation of the brain reward system, among which the nucleus accumbens (NAc) plays a critical role. The mechanism of activation of the NAc neurons is concerned with triggering of the signaling pathways related with the transcription factors like the cyclic AMP-response element binding protein (CREB) and DeltaFosB, as well as enzymes like extracellular signal-regulated kinase (ERK). In this study we compared the activation patterns of above-mentioned markers in the neurons of NAc of the rat after stimulation with nicotine and caffeine. Our results reveal apparent differences in the activation patterns of the studied markers in the NAc evoked by both psychostimulants. These results may be useful for explanation of some behavioral reactions elicited by each of the studied substances and for explanation of developing addiction.

|A37|

Neuropathic pain alters gene transcription in the nucleus accumbens

Wawrzczak-Bargiela A., Bilecki W., Rojewska E., Mika A., Ziolkowska B., Przewlocka B., Przewlocki R.

Department of Molecular Neuropharmacology, Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland
bargiela@if-pan.krakow.pl

Nucleus accumbens, which is an important component of the mesolimbic dopaminergic reward system, also plays a role in pain. However, the molecular mechanisms of this involvement remain unknown. In the present study we explored molecular pathways involved in the development of neuropathic pain. This may allow finding biomarkers for expression of neuropathic pain-like behavior and indicating brain regions involved in this process.

Neuropathic pain was induced by applying a Chronic sciatic nerve Constriction Injury (CCI) model in C57BL/6J mice. Two behavioral tests for neuropathic pain were used: the von Frey's test to measure mechanical allodynia and the cold plate test to assess thermal hyperalgesia.

Using qRT-PCR analysis, we found that nerve injury produced a significant increase in the expression of opioid propeptide prodynorphin and proenkephalin genes (PDYN, PENK), opioid kappa and delta receptors genes (KOR, DOR) and calcium/calmodulin-dependent protein kinase kinase 1 (CAMKK1) in the nucleus accumbens. Furthermore, we observed that neuropathic pain augmented the expression of stress – and inflammatory response genes coding for the glucocorticoid receptor (GR), FK506 binding protein 5 (FKBP5), and interleukins IL1 beta and IL6 in the nucleus accumbens. Moreover, elevated levels of Glial Fibrillary Acidic Protein (GFAP – astrocyte marker) but not C1q (microglia marker) mRNAs were detected.

Our results demonstrate that CCI produces lasting biochemical changes in a brain region implicated in mood regulation, reward learning and motor function. Taking into account the well-known roles of opioid systems in pain transmission and emotional processes, the observed changes in the expression of the opioid propeptides and receptors genes may contribute to changes in pain sensitivity and in affective response to nociceptive stimulation underlying the development of neuropathic pain. Furthermore, increased expression of GFAP, GR, FKBP5, IL-6 and IL-1beta genes suggests that cellular stress and inflammatory processes are involved in this type of pain not only on the level of the spinal cord but also in the brain.

Acknowledgement: research supported by HEALTH-F2-2013-602891 NEUROPAIN.

|A38|

Effects of chronic restraint stress and betaxolol treatment on p(Ser845)GluA1, GluA1, Arc and beta(1)adrenergic receptor levels in rat hippocampus

Wiatrowska K., Zelek-Molik A., Kowalska M., Nalepa I.

Department of Brain Biochemistry, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

Studies in humans and rodents have demonstrated that chronic stress adversely affects physiological functions and neuronal structure of the hippocampus (HP). In addition to corticosteroids, stress causes rapid release of noradrenaline in some brain areas, which can regulate neuronal function in HP by means of the adrenergic receptor. High level of noradrenaline results in stimulation of beta adrenergic receptors (beta-AR) leading to activation of protein kinase A (PKA) and phosphorylation at Ser845 of GluA1 subunit of AMPA receptors (AMPA-R). This augments synaptic insertion of AMPA-R. The opposite process – the down-regulation of AMPA-R at postsynaptic sites, depends on the activity-regulated cytoskeleton-associated (Arc) protein which enhances the basal rate of receptor endocytosis. We investigated the influence of stress procedure on the p(Ser845)GluA1, GluA1, Arc and beta(1)-AR levels in rat hippocampus and evaluated whether the blockade of beta(1)-AR with specific antagonist, betaxolol, during stress can modulate observed changes.

Male Wistar rats underwent the chronic restraint stress applied for 3 hours daily, for 14 days. During the last 7 days rats were treated with betaxolol (5 mg/kg p.o.) given immediately after daily stress. Next day after a completion of stress procedure the rats were decapitated, their hippocampi were dissected and the extracted proteins were assessed by standard Western blot analysis.

Chronic restraint stress increased phosphorylation of GluA1 at Ser845, with no changes in its total protein level. Betaxolol treatment did not change the stress-induced effect. Also there was no effect of stress and betaxolol treatment alone or in combination on the protein expression of beta(1)-AR and Arc.

The increased phosphorylation at Ser845 of GluA1 with no changes in total GluA1 and Arc protein levels suggests that prolonged stress augments the AMPA-R activity in hippocampal postsynaptic membranes. Phosphorylation at Ser845 of GluA1 results from PKA activation which depends on the beta(1)-AR activity. The lack of modulatory effect of beta(1)-AR blockade during stress on the level of p(Ser845)GluA1 together with the unchanged expression of beta(1)-AR observed in our study indicate that stress-induced change of AMPA-R activity in rat HP was not mediated by beta(1)-AR.

Supported by statutory funds of the Institute of Pharmacology, Polish Academy of Sciences.

|A39|

The apoptotic effects of chemical UV-filter benzophenone-3

Wnuk A., Rzemieniec J., Kajta M.

Department of Experimental Neuroendocrinology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

wnuk@if-pan.krakow.pl

Introduction: Benzophenone-3 (BP-3) is one of the most widely used chemical UV filter that has been available as a sunscreen agent for over 40 years. BP-3 is used as an active ingredient at levels of up to 10% in Europe. Moreover, BP-3 is accepted by the FDA as an indirect food additive. Chemical filters are generally used in combination because no single active agent, used at levels currently permitted by legislation, would provide sufficient protection against UV. Recent epidemiological data demonstrated a strong correlation between prenatal exposures to BP-3 and abnormal innervation of peripheral tissues as observed in 3-4 year old children. However, knowledge about the effect of BP-3 on the central nervous system is limited.

Objectives: This study aimed at clarifying molecular mechanisms of BP-3 action on brain neuronal cells with particular focus on apoptosis which is strongly associated with brain development.

Methodology: Primary neuronal cell cultures, measurements of caspase-3, ROS formation, microarrays and Hoechst 33342 staining were performed as previously. Mouse primary neocortical cell cultures were exposed to BP-3 (1-100 μM) for 6-24 h.

Results: Our study demonstrated that BP-3 (25-100 μM) induced intrinsic apoptosis pathway in the mouse neocortical cell cultures. It was evidenced by 31% loss of the mitochondrial membrane potential and approx. 90-120% rise of

ROS formation and caspase-3 activity. These effects were accompanied by apoptotic fragmentation of cell nuclei as detected by Hoechst 33342 staining. Using specific inhibitors, we provided evidence that BP-3-induced apoptosis of embryonic neuronal cells not only is caspase-3-dependent, but also is a caspase-9-, GSK-3β- and p38/MAPK-mediated process. Furthermore, by the use of microarray analyses we demonstrated BP-3-induced upregulation of apoptosis related genes such as Bax, Bak, Bad.

Conclusion: Summing up, our study has demonstrated that BP-3 induces apoptosis in mouse neuronal cell cultures, that position this UV filter as a risk factor for neurodevelopmental abnormalities.

This study was supported by the NCN no. 2014/13/N/NZ4/04845 and the statutory fund of the Institute of Pharmacology PAS, Crakow, Poland.

A. Wnuk and J. Rzemieniec are scholars of KNOW sponsored by Ministry of Science and Higher Education, Poland.

|A40|

Cardiovascular and respiratory effects of endomorphin-2 injection into the femoral vein of anaesthetized rat

Wojciechowski P., Kaczynska K.

Laboratory of Respiration Physiology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
pwojciechowski@imdik.pan.pl

Introduction: Endomorphin-2 (EM-2) is abundant in central and peripheral nervous system i.a. in the regions involved in pain transmission and respiratory and cardiovascular regulation. It presents high affinity and selectivity towards mu-opioid receptors. The sparse information on EM-2 action on respiratory system is focused primarily on the changes in the ventilation rather than in the shape of the respiratory pattern. The aim of this study was to determine the effects induced by intravenous challenge of endomorphin-2 on cardiovascular and respiratory variables.

Methods: Cardiovascular and respiratory parameters (blood pressure, heart rate, tidal volume, respiratory frequency and minute ventilation) were measured in 21 Wistar rats anaesthetized with 750 mg/kg of urethan and 150 mg/kg of a-chloralose. Animals breathed spontaneously room air via tracheal tube and were treated with an intravenous injection of endomorphin-2 (1 mg/kg) in the following scheme: in control conditions (n = 6); after bilateral dissection of cervical vagi nerves (n = 5); after blockade of opioid receptors with antagonist active in the periphery – naloxone methiodide (2 mg/kg) (n = 5); following blockade with blood-brain barrier penetrant – naloxone hydrochloride (2 mg/kg) (n = 5).

Results: Bolus injection of 1 mg/kg of endomorphin-2 into the femoral vein evoked an apnoea of mean duration of 5.9 ± 1.8 s. After the cessation of breathing tidal volume remained similar to the baseline value. Transiently diminished respiratory frequency failed to have an impact on the minute ventilation. EM-2 challenge caused marked hypotension and transiently slowed down the heart rate. All cardiovascular and respiratory effects of EM-2 were abolished by midcervical vagotomy as well as by blood-brain barrier penetrating (naloxone hydrochloride) and nonpenetrating (naloxone methiodide) opioid receptor antagonist.

|A41|

Diversity in regulation of cell cycle and apoptotic response between familial and sporadic Alzheimer's disease lymphocytes

Wojsiat J.[1], Alquezar C.[2], Laskowska-Kaszub K.[1], Martin Requero A.[2], Wojda U.[1]

[1]Laboratory of Preclinical Testing of Higher Standard, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Cellular and Molecular Medicine, Centro de Investigaciones Biológicas (CSIC), Madrid, Spain, and CIBER de enfermedades raras (CIBERER)

Alzheimer's disease (AD) was first described over 100 years ago. It is the most common cause of dementia with an estimated prevalence of 30 million people worldwide. The growing body of data have shown that AD is characterized by complex alterations in cellular processes that occur not only in neurons, but also in peripheral cells such as lymphocytes. Our previous studies have demonstrated that lymphocytes from sporadic form of AD (SAD) show G1 phase arrest and increased levels of p21 protein, the key regulator of apoptosis and G1/S cell cycle checkpoint. Since it is known that p21, besides controlling the G1/S checkpoint, can regulate apoptosis, we decided to investigate whether p21 levels play a role in the cellular response to an oxidative stress challenge like 2d-ribose (2dRib) treatment. We report here that cells from familial AD (FAD) are more resistant to 2dRib-induced cell death than control or SAD cells. Quantitative real-time PCR reactions and Immunoblotting experiments showed that p21 mRNA and protein levels

significantly increased in FAD cells in response to 2dRib. In addition, using cell fractionation and confocal microscopy imaging, we found a higher cytosolic accumulation of p21 in FAD cells. Transcriptional activation of p21 was shown to be dependent on p53, as it can be blocked by PFT-a and was correlated with phosphorylation of p53. Thus in human B-lymphocytes under oxidative stress evoked by 2dRib, 7 PS1 mutants seem to strongly exacerbate phosphorylation of p53 exhibiting gain of function effect over wtPS1. This activities of mutPS1 seem to represent a compensatory mechanism against acute oxidative stress, preventing depolarization of mitochondrial membrane and apoptosis in human FAD lymphoblasts. Altogether, our results showed that mechanism of apoptotic response to acute oxidative stress distinguishes cells from SAD and FAD patients. Thus, caution should be taken in extrapolating data obtained from cellular or animal models based in FAD mutations, as they may not be relevant in SAD. Consistently, therapeutic designs should take into account the possible effect variability in SAD versus FAD cells.

|A42|

Eplerenone affects kynurenic acid production in rat brain cortex *in vitro*

Zakrocka I.[1], Turski W.A.[2], Kocki T.[3]

Medical University of Lublin, Department of Experimental and Clinical Pharmacology, Lublin, Poland

Arterial hypertension is the predominant cause of cardiovascular disorders in humans. The most recommended antihypertensive drugs are inhibitors of renin-angiotensin-aldosterone system (RAAS), which showed great improvement in prolonging patients survival. RAAS plays an important role in blood pressure regulation both in the central nervous system and peripheral tissues. Eplerenone is a mineralocorticosteroid antagonist with diuretic and potassium sparing effect. In recent studies was shown that eplerenone reverses enhanced brain glutamatergic signaling in animal models with arterial hypertension.

Kynurenic acid (KYNA) is the endogenous antagonist of glutamate receptors and of α7 nicotinic receptors. Cerebral synthesis of KYNA from L-kynurenine (L-KYN) is catalyzed by kynurenine aminotransferases (KAT) localized mainly in astrocytes. KYNA has neuroprotective properties and its impaired production was implicated in various neurodegenerative diseases. Additionally, it was shown that KYNA plays a role in the regulation of blood pressure after local intracerebral administration.

The aim of this study was to evaluate the effect of eplerenone on the KYNA synthesis in rat brain cortex *in vitro*.

Experiments were conducted on male Wistar rats brain cortex. The effect of eplerenone on KYNA concentration and KATs activity was evaluated on cortical slices and cortical homogenates after 2 hours of incubation with L-KYN and different concentrations of tested drug. Eplerenone at the concentration of 0.01 mM and 0.1 mM didn't change the KYNA synthesis in the brain cortical slices. At the concentration of 0.5 mM eplerenone decreased KYNA production in the brain cortical slices to 90% of control. The activity of KAT I was decreased by eplerenone at the concentration of 0.01 mM, 0.05 mM, 0.1 mM and 0.5 mM to 92%, 71%, 69% and 61% ($p < 0.05$) of control value, respectively. The activity of KAT II was diminished by eplerenone at the concentration of 0.01 mM, 0.05 mM, 0.1 mM and 0.5 mM to 84%, 50% ($p < 0.001$), 49% ($p < 0.001$) and 45% ($p < 0.001$) of control, respectively.

Presented data suggest that eplerenone might have an influence on KYNA production in rat brain cortex, most probably by changing KAT's activity.

Supported by the grant from National Science Centre (NCN) PRELUDIUM 4 No UMO-2012/07/N/NZ4/02088.

|A43|

SNAP-25: potential link between genetic and environmental causes of autism

Zieminska E., Lenart J., Lazarewicz J.W.

Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
elziem@imdik.pan.pl

Autism spectrum disorder (ASD) is a severe neurodevelopmental disability disrupting the behavior of patients. Its unclear etiology most likely involves both genetic and environmental factors. Many substances are suspected of causing ASD. SNAP-25 (synaptosomal associated protein, 25 kDa), which encodes a presynaptic protein involved in neurotransmitter release and the modulation of voltage-gated calcium channels, is a candidate gene that is implicated in neuropsychiatric disorders, including ASD. The aim of this *in vitro* study, which was performed on a primary culture of rat cerebellar granule cells (CGCs), was to test the hypothesis that suspected developmental neurotoxins can alter SNAP-25 mRNA and protein expression. The test substances included environmental toxins: tetrabromobisphenol-A (TBBPA), the organomercury com-

pound thimerosal (TH), and silver nanoparticles (NAg). Teratogens: valproic acid (VPA) and thalidomide (THAL), that cause behavioral changes similar to autism in rodents were also tested. The substances were administered to CGC cultures for 24 h at subtoxic concentrations. Obtained results demonstrated that SNAP-25 mRNA levels were increased by 49 and 66% by TBBPA and THAL, respectively, whereas VPA and NAg reduced levels to 48 and 64% of the control, respectively. In addition, the SNAP-25 protein content in CGCs was increased by 79% by TBBPA, 25% by THAL and 21% NAg, whereas VPA and TH reduced SNAP-25 level to 73 and 69% of the control, respectively. Thus, this study is the first to demonstrate the effect of different compounds that are suspected of causing autism on SNAP-25 expression; the results suggest that this protein may be a common target for not only inherited but also environmental modifications linked to ASD.

This study was supported by the Polish National Science Centre; grant numbers 2012/05/B/NZ7/03225 and 2014/15/B/NZ4/04490.

|A44|

Expression regulation of opioid propeptides and dopamine receptors in the mesostriatal system by chronic intake of palatable foods

Ziolkowska B.[1], Gieryk A.[1], Martín-García E.[2], Burokas A.[2], Maldonado R.[2], McDonald J.[3], Dierssen M.[3], Przewlocki R.[1]

[1]Department of Molecular Neuropharmacology, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland
[2]Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona, Spain
[3]Genes and Disease Program, Genomic Regulation Center-CRG, Pompeu Fabra University, Barcelona, Spain

Chronic (self-)administration of addictive drugs typically up-regulates the prodynorphin (PDYN) gene expression in the dorsal striatum or nucleus accumbens. Drug effects on transcription of other genes in the mesostriatal system, including those coding for proenkephalin (PENK) and dopamine eceptors, have also been characterized. The aim of our study was to determine if prolonged intake of highly palatable foods, which may produce addiction-like behaviours, affects the mesostriatal gene expression in the same manner as drugs of abuse.

Two models of chronic intake of palatable foods by C57BL/6 mice were used: 1) instrumental self-administration of cocoa-flavoured pellets (daily 1-h sessions for 20 days) and 2) diet-induced obesity (DIO) model, i.e. constant free-choice access to a chocolate-based diet for 10 weeks. mRNA levels of PDYN, PENK and the dopamine receptors D1 and D2 (D1R, D2R) were assessed in the mesostriatal system by *in situ* hybridization.

We demonstrated that the PDYN mRNA levels were down-regulated in the dorsal striatum and nucleus accumbens in the instrumental model, and in the central amygdala of DIO mice. The D2R transcript was down-regulated in the nucleus accumbens both in the instrumental self-administration and DIO model. Expression of PENK and D1R remained unchanged in both models.

In conclusion, neuroadaptations in the mesostriatal system produced by chronic intake of palatable foods seem distinct from those produced by drugs of abuse. Whereas tasty food self-administration fails to up-regulate the PDYN gene (unlike addictive substances), its characteristic effect seems to be down-regulation of the D2R in the nucleus accumbens, particularly in the shell (not shared by drug abuse models). This suggests that drug and food addictions involve different neuronal mechanisms.

This work was supported by the EU grant LSHM-CT-2007-037669 (PHECOMP), the National Science Centre (Poland) grant 2013/08/A/NZ3/00848 and by the statutory activity funds from the Institute of Pharmacology, Polish Academy of Sciences.

Poster session II

|B1|

Implementation of human induced pluripotent stem cells (hiPSC) to test DNT at the earliest stages of development

Augustyniak J., Zychowicz M., Winiarska H., Buzanska L.

Stem Cell Bioengineering Laboratory, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Human induced pluripotent stem cells (hiPSC) hold similar properties as human embryonic stem cells (hESC), however are not ethically controversial and easy to obtain. They possess ability to form embryoid bodies (EBs). EBs are three-dimensional aggregates of pluripotent stem (PS) cells which can recapitulate the early development of human embryo, thus can serve as the alternative model for toxicity and drug testing of neurodevelopmental toxic compounds. Methylmercury chloride (MeHgCl) is a developmental neurotoxin with strong embryotoxic effect for human embryo. In mouse *in vitro* culture cells model it has been defined as weakly embryotoxic or not embryotoxic. Here we present that methylmercury chloride is strongly embryotoxic for hiPSCs that are cultured as 3D embryoid bodies' stage of development during neural commitment. Comparison of the MeHgCl effect on three hiPSC different stages of development: non-differentiated hiPS cells in monolayer, the embryoid bodies (3D culture) and hiPS cells neurally committed, showed that the susceptibility to tested compound is developmental stage dependent. The three-dimensional aggregates were more sensitive than hiPSC after direct induction of neuronal differentiation. We have also confirmed inhibition of the ability of hiPSC to form embryoid bodies after 24 h of MeHgCl exposition. The increase in free radicals and reduction of the mitochondrial membrane potential were accompanied by a reduced cell viability after MeHgCl treatment. The obtained results indicate that hiPSC can be considered as an in vitro model for testing human embryotoxicity and developmental neurotoxicity replacing mouse embryonic stem cell (EST) test.

The work is supported by National Science Centre via Grant No 2013/11/B/N21/00089 and DEC-2011/03/B/ST8/05867 EU co-financed under OPHC and statutory funds to MMRC.

|B2|

Human breast milk as a new source of stem/progenitor cells

Dzwigala M.E.[1,2], Zychowicz M.[2], Romejko-Wolniewicz E.[1]

[1]2nd Department of Obstetrics and Gynecology, Medical University of Warsaw, Poland
[2]Translative Platform for Regenerative Medicine, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

monica.dzwigala@gmail.com

Introduction: Human breast milk is a well-known rich source of nutrients and immunological factors for a newborn. Some studies indicate the presence of stem/progenitor cells in that source. The aim of this study is to confirm the presence of human breast stem cells (hBSC) in human breast milk and to evaluate pluripotent and regenerative potential of isolated cells.

Material and methods: Samples of breast milk were obtained in 0 to 4 day post-delivery from mothers after natural delivery or cesarean section in different gestational age. The isolation of cells was performed up to 4 hours since collection of the sample. Milk sample was diluted with equal volume of sterile phosphate buffered saline (PBS) and centrifuged. The fat layer and supernatant were removed, and obtained cell pellet was washed three times in PBS. Cells viability and concentration were estimated in a Burker chamber by Trypan Blue exclusion. Various types of media were tested: DMEM + 10% FBS, Essential 8 medium, CellGro medium, MammoCult. hBSC were seeded in low adherent plates or standard culture plates. The cells were characterized by immunofluorescence technique (IF) and reverse transcription polymerase chain reaction (RT-PCR).

Results: In our study we succeed in isolating stem-cell like population. Our results indicate that breastmilk cells from different donors displayed variable expression of pluripotency genes normally found in human embryonic stem cells (hESCs). These genes included the transcription factors (TFs) OCT4, SOX2, NANOG, known to constitute the core self-renewal circuitry of hESCs. The RT PCR data have also revealed the presence of pluripotency markers (OCT4, SOX2, NANOG) in isolated cells.

Conclusions: Stem/progenitor cells (SC) can be obtained from the human breast milk. The human breast milk can be a new noninvasive, needle free source of hBSC. The hBSCs can be considered to become a new, alternative source of SC and new standard for SC-based therapies.

The work is supported by statutory funds to 2nd Department of Obstetrics and Gynecology Medical University of Warsaw and Ministry of Science and Higher Education funds.

|B3|

Lowered oxygen concentration *in vitro* promotes and stabilizes mesenchymal ADRC phenotype and genotype for safe clinical application

Figiel-Dabrowska A.[1], Obtulowicz P.[1], Domanska-Janik K.[1], Sarnowska A.[1,2]

[1]Stem Cell Bioengineering Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Translative Platform for Regenerative Medicine, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

The number of mesenchymal stem cells (MSC)-based therapies is increasing year by year. Numerous independent studies confirmed therapeutic effectiveness of mesenchymal type of Adipose-Derived Regenerative Cells (ADRC) in various clinical settings. Nonetheless broad distribution and easy surgical accessibility of adipose tissue, as well as frequently observed heavy deterioration of patients' general conditions and needs of high number of autologous cells for mono- or multiple transplantations, force elaboration of safe and enhanced method of these cells' expansion in vitro. Previously, it was confirmed that oxygen concentration is one of the most important determinants of stem/progenitor cell metabolism, viability, proliferation and differentiation, leading to the enormous amplification of the MSC amount in culture.

The aim of this study was to characterize and confirm stability of ADRC phenotype and genotype during a long term cell culture expansion in 21% vs lowered to 5% oxygen concentrations. The ADRC were isolated by CellCelution System by commercial method performed in typical clinical surrounding. The MSC-like phenotype and ability to multi-lineage differentiation were assessed. Proliferation of cultures was controlled steadily by increased cell density and expressed as the population doubling time (PDT) and the cumulative population doublings (CPDs). The morphological control of ADRC phenotype stability and cell senescence were carried out during long term cultivation in both examined oxygen conditions by the use of typical immunocytochemical methods. The cells growing until 30th passage additionally underwent chromosome analysis – karyotyping.

Our results expressly indicate that the low oxygen "physioxic" culture conditions significantly favour ADRC long-term proliferation and well preservation of their phenotype and genome stability. The significant enhancement of the aforementioned low oxygen dependent ADRC properties in culture can positively influence their further therapeutic effectiveness.

The work was supported by National Science Centre grant No 2011/01/B/NZ3/05401 and National Centre for Research and Development grant No Strategmed 1/234261/2/NCBR/2014.

|B4|

Effects of sex hormones on apoptosis in cells with mutations responsible for Leber's hereditary optic neuropathy

Jankauskaitė E.[1], Kodron A.[1], Bartnik E.[1,2]

[1]Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
[2]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Leber's hereditary optic neuropathy (LHON) is the most common mitochondrial disease resulting in central vision loss due to optic nerve atrophy. Most cases are caused by a mutation in mitochondrial genes encoding one of three subunits of mitochondrial complex I (ND1, ND4, and ND6). LHON is considered as a disease of young men because of its early onset between 20 and 30 years and male preponderance (50% men and only 10% women with mutations predisposing to LHON are affected). The mtDNA usually is homoplasmic for the mutation in every cell. Changes in mitochondrial respiratory chain or ROS production can lead to induction of apoptosis. Most LHON mutations involve a decrease in Complex I activity and some data suggest that cells with these mutations are more prone to apoptosis. In females, estrogens are thought to modify the severity of mitochondrial dysfunction, including defective ATP synthesis, oxidative stress, and apoptosis (Giordano *et al*., 2011). However, no increase of LHON in post-menopausal women is observed. Estrada *et al*. (2006) reported that high concentrations of testosterone initiate an apoptotic pathway and induce neurotoxicity in neuroblastoma cells. So far not much data has been published regarding the role of testosterone in apoptosis in LHON affected men. The objective of this research is to determine the effect of sex hormones on cell death in LHON patient and

control cells. Human optic nerve cells are unavailable for studies, therefore a lymphoblast cell model was used. 4 cell lines from a unique Polish family harboring two LHON mutations m.11778G>A and m.3460G>A and healthy controls were enrolled into this study. The effect of testosterone on initiation of apoptosis was investigated via the canonical apoptotic pathway which involves activation of aspartate-specific proteases (caspases). Apoptosis was confirmed by PARP cleavage. Here we present for the first time the influence of the sex hormone testosterone on apoptosis in LHON. We did not observe any differences in level of apoptosis in the LHON patient and control cells. However, in the control cells apoptosis was activated via a caspase dependent pathway, while in LHON cells apoptosis was initiated by a different, caspase independent pathway.

|B5|

Effect of culture conditions on proliferation and differentiation of rat oligodendrocyte progenitor cells

Janowska J., Sypecka J.

NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

jjanowska@imdik.pan.pl

Background: Oligodendrocyte progenitor cells (OPCs) can give rise to myelinating oligodendrocytes but they are also involved in modifying neural microenvironment, e.g. in response to damaging conditions. It is crucial for studies of the biology of OPCs in vitro to culture them at physiological normoxia (2-5% O_2) and to have the possibility of tracing their paracrine activity in a defined, serum-free medium. The aim of our study was to evaluate growth of rat OPCs in vitro in different culture conditions which may influence cell proliferation and maturation.

Material and methods: Mixed glial cell cultures were established from brain hemispheres of 2-day-old Wistar rat pups and plated in a tissue culture flask coated with poly-L-lysine. The cells grew under standard conditions in DMEM containing 10% FBS for 12 days. OPCs were obtained from mixed glial cell cultures with a shaking method which uses differential adherent properties of glia. The isolated OPCs were then plated at densities between 1.2 and 6×10^4 cells/cm^2 and cultured under 5% or 21% O_2 either in serum-free medium or supplemented with 1% FBS. After 2 DIV or 5 DIV the cells were fixed and identified by immunolabeling with antibodies against NG2 for OPCs, GalC for immature oligodendrocytes, MBP and PLP for myelinating cells, and Ki67 for proliferating cells.

Results: The OPCs plated at higher density proliferated more intensely, but the process of their maturation was significantly slowed-down. The cells seeded sparsely had more complex morphology and were able to express myelin proteins even only after 2 days in culture. The 1% FBS addition to culture medium enhanced OPC differentiation at low oxygen level, but had no influence on the number of GalC- and MBP-positive cells at 21% O_2 level.

Conclusions: Although a low concentration of FBS in a culture medium appreciably promotes oligodendrocyte maturation, the OPC differentiation proceeds correctly through its typical stages in physiologically-relevant conditions created in vitro. Application of serum-free media and normoxic oxygen tension provides a good model to analyze active compounds OPC release during growth in well-defined conditions.

Supported by NCN (National Science Centre, Poland) grant no. 2014/15/B/NZ4/01875.

|B6|

A histone deacetylase inhibitor, Sodium Butyrate, prevents an inflammatory response associated with neonatal hypoxia-ischemia

Jaworska J., Ziemka-Nalecz M., Zalewska T.

NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

jjaworska@imdik.pan.pl

Neonatal hypoxia-ischemia (HI) causes considerable mortality and long-term neurological deficits. Mounting evidence indicate that administering histone deacetylase inhibitors (HDACis) in adult animal stroke models provides neuroprotection, associated with reduced neuroinflammation. The aim of our study was to examine the effect of Sodium Butyrate (SB), a HDACi, on inflammatory processes after neonatal HI injury.

We used a model of HI induced in 7-day old rats. The left common carotid artery was ligated and animals were exposed to hypoxia (7.6% oxygen for 60 min). The hypoxic undamaged hemisphere and sham-operated rats served as control. SB was injected subcutaneously for 5 days starting immediately after HI. Microglial cells and astroglia were identified immunohistochemically with specific markers

(ED1 and GFAP, respectively). Additionally, we determined the expression of transcription factor NFkappaB and pro-inflammatory protein COX-2 using Western Blot, whereas the level of other pro-inflammatory factors (IL-1alpha, IL-1beta, IP-10, TNFa) were estimated by magnetic bead–based assay (Luminex).

HI markedly increased the density of microglia (ED1+) in the ipsilateral hemisphere which was further enhanced by SB application. A significant number of microglial cells after inhibitor treatment presented an anti-inflammatory M2 phenotype (ED1+/Arg1+). At the same time suppression of pro-inflammatory M1 cells (ED1+/IL-1B+) was observed. SB treatment also resulted in an over 2-fold elevation in the GFAP staining intensity in the ipsilateral injured hemisphere (HI+SB) however the amount of astroglial cells expressing IL-1β within the cortex of the damaged ipsilateral hemisphere was markedly reduced upon SB administration. Moreover, treatment with the inhibitor decreased the level of NFkappaB and pro-inflammatory factors (COX-2, IL-1beta and IP-10).

This study provides evidence that inhibition of HDACs by SB exhibits beneficial anti-inflammatory action.

Supported by National Science Centre, Poland grants no. 2012/05/B/NZ3/00436 and 2015/17/N/NZ7/00969.

|B7|

Effect of myriocin and FTY720 on gene expression of sphingolipid enzymes in mouse model of Alzheimer's disease

Jesko H.[1], Wencel P.L.[2], Strosznajder R.P.[2]

[1]Department of Cellular Signalling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Laboratory of Preclinical Research and Environmental Agents, Department of Neurosurgery, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Silgrin@interia.pl

Background: Sphingolipids modulate neurotransmission, cell survival, and may modify amyloid beta (AB) toxicity in Alzheimer's disease (AD). Accumulation of pro-apoptotic ceramide (Cer) and loss of the protective sphingosine-1-phosphate (S1P) parallel the neurodegeneration and might modulate AB production. Deciphering the early AD mechanisms is of greatest importance: the long initial period of relatively asymptomatic neurodegeneration makes AD extremely difficult to diagnose before irreversible damage occurs.

Objectives: We examined the influence of sphingolipid-modulating compounds (myriocin and FTY720) on gene expression of enzymes metabolizing sphingosine/S1P, sphingomyelin, Cer/Cer-1-phosphate in streptozotocin (STZ) induced AD model.

Methodology: Intracerebroventricular administration of STZ was used to induce the neurodegenerative symptoms characteristic for the prevailing sporadic AD in C57Bl6/J mice. Animals were intraperitoneally treated for 14 days with myriocin (serine palmitoyltransferase inhibitor) and FTY720 (S1P receptor modulator). Gene expression levels in brain cortex and hippocampus were assessed using Real-time PCR.

Results: In the brain cortex, treatment with STZ and myriocin significantly elevated mRNA expression of sphingomyelin phosphodiesterases Smpd1 and Smpd2, and ceramide kinase, as compared to STZ+vehicle-treated controls. Thus, although myriocin inhibits de novo ceramide synthesis, elevated SMPDs should counteract the resulting drop in cortical Cer level while the kinase would increase ceramide phosphate. Administration of FTY720 together with STZ led to tendency towards reduction of cortical SMPDs. We also observed that hippocampal mRNAs of SMPDs and Cer kinase remained unchanged in STZ+myriocin and STZ+FTY720 treated animals. S1P phosphatase 1 (Sgpp1) was reduced in the hippocampus of FTY720 treated animals and showed a similar tendency in the cortex, probably facilitating the maintenance of S1P levels.

Conclusions: Our results show that myriocin and FTY720 exert significant effects on the expression of genes related to ceramide metabolism and S1P signaling. Myriocin tends to increase ceramide kinase expression while FTY720 appears to exert its influence in two ways, through the reduction of SMPDs, and Sgpp1. Changes in cellular sphingolipids may affect the metabolism of AB and its precursor which subsequently could impact cell survival and death.

Supported by the National Science Center (http://www.ncn.gov.pl) grant no. NCN/15/B/NZ3/01049.

|B8|

Profile of surface glycans in subpopulations of mesenchymal stem cells

Jezierska-Wozniak K.[1,2], Wojtkiewicz J.[2,3,4], Maksymowicz W.[1,2]

[1]Department of Neurology and Neurosurgery, Faculty of Medical Sciences, University of Warmia and Mazury, Olsztyn, Poland
[2]Laboratory for Regenerative Medicine, Faculty of Medical Sciences, University of Warmia and Mazury, Olsztyn, Poland
[3]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Poland
[4]Foundation for the Nerve Cells Regeneration, Olsztyn, Poland

The most common definition of mesenchymal stem cells says that mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes and adipocytes. However, it is huge oversimplification, because only one-third of the MSC clones generated from bone marrow mononuclear cells are able to differentiate in trilineage potential suggesting that MSCs are constituted by a group of cells with different differentiation potential. Described variation relates to cells isolated from different sources and relate to the potential for differentiation. Results based on the analysis of real-time PCR and flow cytometric assay indicates that one of the most homogeneous fractions of MSCs is a sub-population of cells present on their surface marker CD271. It is characterized by high potential to differentiate into cells of the bone and cartilage, and is the most abundant source of stromal bone marrow. It is thought that a large variety of glycans closely located on the surface of the cell is important because of their specific interactions that control cell-cell adhesion, immune response or microbial pathogenesis. The structure of surface glycans reflects cellular changes, such as cell-cycle, differentiation, and various conditions of its physiological activity. All of these states are indicated on the cell surface with changes in the cell membrane which decorates glycoproteins and glycolipids. This is possible due to huge amount of – estimated at nearly 2000 – glycogen, or programming protein genes responsible for the synthesis of proteins responsible for the synthesis of glycan processing and transport structural. We performed a comparative analysis of surface glycans in the entire population MSCs and two sub-populations, possitive and negative for CD271 marker using a lectin panel specific for oligosaccharides present in human cells. Taking into account the role of glycans in different states of the cell, we need to be aware of the fact that they glycans will be extensively utilized and critically contribute to the use of stem cells for future therapeutic transplantation. In the successful cell therapy it is essential to establish standardized multi-step protocols to generate high quality and efficacy therapeutic biomaterial.

|B9|

Evaluation of the changes in the cell surface glycan profiles in the population of mesenchymal stem cells in response to the stimulation with pulsed electric field

Jezierska-Wozniak K.[1,2], Barczewska M.[1], Wojtkiewicz J.[2,3,4], Maksymowicz W.[1,2]

[1]Department of Neurology and Neurosurgery, Faculty of Medical Sciences, University of Warmia and Mazury, Olsztyn, Poland
[2]Laboratory for Regenerative Medicine, Faculty of Medical Sciences, University of Warmia and Mazury, Olsztyn, Poland
[3]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Poland
[4]Foundation for the Nerve Cells Regeneration, Olsztyn, Poland

One of the major components of the cellular membrane that define cell-cell and cell extracellular matrix interactions is the cell glycome consisting of a large variety of complex glycans covalently attached to the membrane proteins and lipids. These glycoproteins and glycolipids are actively involved in, and often control cell-cell signaling, microbial infections, immune response, wound healing, and other events on the cellular and tissue levels. The dynamics of a glycomic profile reflects changes in the cellular activities such as division, differentiation, motility, secretory functions and malignant transformation. Glycoconjugate-targeting investigations would therefore be expected to streamline discovery of novel biological factors of potential clinical significance. With the aim of developing novel techniques to analysis of cell movement, we tested whether the system of deep brain stimulation contributes to creating changes in the cell surface glycan profiles. We performed a comparative analysis of surface glycans in mesenchymal stem cells population after pulsed electric field stimulation using a lectin panel specific for oligosaccharides present in human cells. On the basis of staining with fluorescein-labeled lectins, we have determined the presence of both terminal and internally linked α1-3/α1-6-Fuc, (a-1,3) and (a-1,6) linked mannose, sialic acid attached to terminal galactose in (a-2,6), alpha-linked mannose, terminal GlcNAc, and a-linked mannose. After electrical field stimulation changes were observed for all lectins. They concerned both signal intensity and

placement. Analysis of a panel of lectins, in order to evaluate changes in the profile of surface glycans revealed significant changes in the expression of cell surface glycans. We used glycans staining following hypothesis that application of different lectins highlighting different types of glycans in human cells. That would allow observed dynamics of cell surfaces reprogramming glycosylation in cell populations under the influence of the electric field of different values. Obtained results made possible to assess the impact of the electric field on the degree of cell migration and changes in cell phenotype and allowed choosing the right parameter of stimulation. We can assume that they can have a significant impact on the quality of the therapeutic preparation.

|B10|

Astrocyte-conditioned medium protecting cholinergic neurons against zinc-induced neurotoxicity

Klimaszewska-Lata J., Gul-Hinc S., Dys A., Ronowska A., Bizon-Zygmanska D., Jankowska-Kulawy A., Bielarczyk H.

Chair of Clinical Biochemistry, Department of Laboratory Medicine, Medical University of Gdansk, Gdansk, Poland

joannaklimaszewska@gumed.edu.pl

Excessive accumulation of zinc in the brain is one of putative factors involved in pathomechanism of cholinergic encephalopathies. Our previous studies have shown that Zn-dependent losses of many enzymes of energy and acetyl-CoA metabolism, is much more harmful to cholinergic neurons than to the other cells, because they consume acetyl-CoA not only for energy production, but also for the acetylcholine synthesis. Astroglia are known to exert several neuroprotective functions through the supply of many metabolites into the neurons. On the other hand, abundant evidence shows that changes in astrocyte function may contribute to neurodegenerative diseases. Therefore, the aim of this work was to find out whether diffusible factors produced by astroglial cells can affect energy metabolism and survival of cholinergic neurons in neurotoxic conditions. SN56 neuroblastoma cells were used as experimental model of cholinergic neurons. To obtain astroglia-conditioning medium (ACM), C6 glioma cells were grown until confluence. After 36 hours the medium was collected as ACM and transferred to the neuronal cultures for the next 24 hours (with or without zinc). We reported that ACM protected cholinergic neurons against the zinc-induced damages. Zn in concentration 0.15 mmol/l brought about loss of neuronal extensions and intracellular connections as well as appearance of blebs and malformations of cell membranes. These alterations were reversed in part by ACM. In response to the same concentration of zinc, the number of non-viable trypan-blue positive cells was significantly reduced from 60 to 20% in the ACM-cultured cholinergic neurons. In the same conditions, the total number of cells increased three times. We also observed that ACM partially reversed the inhibitory effect of zinc on the energy metabolism of cholinergic neurons in the range of aconitase and isocitrate dehydrogenase activity and the total level of acetyl-CoA. These results indicate that diffusible factors released from astrocytes affect neurons energy metabolism and probably this way promote their survival.

Supported by MN 01-0174/08/248 and ST57 GUMed fund.

|B11|

Study of respiratory function in cybrid cells harbouring Leber's hereditary optic neuropathy mutations

Kodron A.[1], Wojciechowska K.[1], Dymkowska D.[2], Zablocki K.[2], Tonska K.[1], Bartnik E.[1,3]

[1]Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
[2]Laboratory of Cellular Metabolism, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland
[3]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Leber's Hereditary Optic Neuropathy (LHON) is the most common mitochondrial disease. LHON is characterized by sudden, painless loss of vision associated with abnormalities of the optic nerve. The disease in over 90% of cases is caused by one of three mutations in the mitochondrial genome: 11778G>A, 3460G>A or 14484T>C. The co-occurrence of two pathogenic mutations responsible for LHON is extremely rare. Here we present for the first time the respiratory function analysis of cybrid cells harbouring a combination of the 11778G>A and the 3460G>A LHON mutations. Cybrid cell lines were constructed by fusion of enucleated fibroblasts, derived from skin biopsies of 3 LHON patients and 3 healthy controls, with ρ0 143B cells. The heteroplasmy level of each mutation was measured by the last cycle hot PCR-RFLP meth-

od. Cellular respiration was measured in four conditions (basal respiration and after addition of respiratory substrates, ATPase inhibitor and OXPHOS uncoupler) with the use of oxygraph (Oroboros-2k). Analysis showed that LHON cybrids had higher rates of respiration and respiratory capacity compared to control cells. Moreover, respiratory capacity increased with the increasing level of the 11778G>A mutation. These data suggest that LHON mutations could force cells to increase their respiratory capacity to overcome the mutation effect. Increased respiratory capacity could be due to mitochondrial biogenesis induction in response to energy stress caused by LHON mutations.

|B12|

Differentiating oligodendrocyte precursor cells produce interleukin-33 which shapes remyelinating lesion microenvironment

Konarzewska K., Wylot B., Niedziolka S., Dabrowski M., Kaminska B., Zawadzka M.

Laboratory of Molecular Neurobiology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Interleukin-33 (IL-33) plays an important role in maintaining organism homeostasis by several potential activities: as an alarmin, traditional cytokine or nuclear factor. Due to its multiple functions, IL-33 has been recently widely studied in pathophysiology of numerous disorders, including multiple sclerosis (MS). MS is an autoimmune disease characterized by central nervous system (CNS) demyelination. Remyelination is a physiological response to demyelination and is led by the oligodendrocyte precursor cells (OPCs) which differentiate within lesion and serve the axons with new myelin sheaths. However, remyelination often fails, which causes long-term neurological problems for the MS patients.

Using well described *in vivo* model of murine spinal cord demyelination and based on global gene expression profiling, we have selected the group of genes, which products could be potentially involve in the process of OPCs differentiation and one of them was interleukin-33 (IL-33). We hypothesize that IL-33 may play a role in activation of OPCs and remyelination outcome. Here, we provide evidence for the significant role of IL-33 in modeling the white matter damage environment and its impact on the axons remyelination. We demonstrate that IL-33 is highly produced in the intact CNS, by a population of oligodendrocyte lineage cells, but neither OPCs nor white matter mature oligodendrocytes. Moreover, using an *in vivo* model of chemically-induced murine spinal cord demyelination, we show that IL-33 is upregulated, produced and secreted by differentiating OPCs in response to the CNS white matter injury. Secreted IL33 alters the functional phenotype of microglia/microphages, which in consequence allows OPC for the complete differentiation. Our results clearly show, for the first time, that interleukin-33 is produced and secreted by activated OPCs, which shapes the lesion microenvironment and finally indirectly supports white matter regeneration.

Studies were supported by National Science Centre grant 2011/03/B/NZ4/02988.

|B13|

Systemic inflammation at different developmental stages reduces numbers of calbindin-immunopositive neurons and their vulnerability to seizures evoked in adulthood

Kosonowska E., Janeczko K., Setkowicz Z.

Department of Neuroanatomy, Institute of Zoology, Jagiellonian University, Cracow, Poland

Background: Calbindin-D28k is one of the major calcium-binding proteins in the brain. It regulates intracellular response against various stimuli and provides neuroprotection against calcium-mediated neurotoxicity. Previous studies have reported the vulnerability of calbindin-positive (CB+) neurons in the dentate granule cell layer of the hippocampal formation in epilepsy. According to previous studies, neuroinflammation may lead to an increase in seizure susceptibility and trigger epileptogenesis. However, emerging experimental evidence indicates that early age inflammation acting as a preconditioning factor may also have protective effects. The aim of this study was to examine long term effects of systemic inflammation induced at different postnatal developmental stages on the CB+ cell population within hippocampal formation in response to status epilepticus evoked in adulthood.

Methodology: Lipopolysaccharide (LPS) was injected intraperitoneally (2 mg/kg b.w.) to Wistar rats on postnatal day 6 or 30 (P06 and P30, respectively). When became two-month-old, the rats which survived inflammation were injected with pilocarpine to evoke status epilepti-

cus and sacrificed 3 days later. Brain sections were then processed for CB immunohistochemistry and CB+ neurons were counted bilaterally within CA1, CA2/3 and DG regions of the dorsal part of hippocampal formation.

Results: LPS injections alone on P06 or P30 caused significant decreases in numbers of CB+ cells but only within the DG area when compared to naïve animals. The epileptic seizures induced in both LPS-untreated controls and LPS-treated animals led to significant decreases of CB+ cell number (vs. naïve animals) but also in the DG. However, in rats injected with LPS on P30 the effect of seizures was significantly lower than in those injected on P06.

Conclusions: Transient inflammation induced during the brain development led to permanent decreases of CB+ neuronal population in the adult brain. The inflammation induced on P30 could also prevent large seizure-related reduction of the cells. This might result from long-term changes in nervous tissue reactivity – preconditioning.

Supported by the NSC grant: UMO-2012/05/B/NZ4/02406.

|B14|

Participation of interleukin 1-β in the axon elongation of dorsal root ganglia (DRG) neurons supplying the urinary bladder of the pig – an *in vitro* study

Kozlowska A.[1], Mikolajczyk A.[2], Majewski M.[3], Majewski M.[1], Klimaschewski L.[4]

[1]Department of Human Physiology, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[2]Department of Public Health, Epidemiology and Microbiology, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Department of Pharmacology and Toxicology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[4]Innsbruck Medical University, Department of Anatomy and Histology, Innsbruck, Austria

kozlowska.anna@uwm.edu.pl

Introduction: Pro-inflammatory cytokines such as interleukin 1-β (IL-1β) are considered to exert detrimental effects during brain trauma and in neurodegenerative disorders. In other way it was reported that IL-1β enhances neurite growth in brain slices. In this study, we analyzed the influence of this cytokine on the axon growth of DRG neurons supplying porcine urinary bladder.

Material and methods: Lumbar and sacral DRGs were collected from 8-10 weeks old pigs (n = 4) which received 2 weeks earlier multiple injections of the Fast Blue retrograde tracer in the wall of the urinary bladder. Cell cultures were prepared as described previously (Hausott *et al*., 2011). Neurons were re-suspended, plated at per glass coverslip coated with poly-D-lysine/laminin and cultivated in TNB medium at 37°C in 5% CO_2 and treated with IL-1β (50 ng/ml, experimental group). After 24 h in culture, neurons were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.01% Triton X-100 in PBS for 5 min and blocked with blocking buffer (10% goat serum in PBS) for 30 min. Cells were incubated with primary antibodies against neuron-specific β-III tubulin diluted in blocking buffer for 1h at room temperature and then incubated with secondary Alexa-488-conjugated antibody for 60 min at RT. Axon length analysis was performed using the WIS-NeuroMath (Rishal *et al*. 2012). The Mann-Whitney U test (GraphPad Software, Inc) was used to analyze the variability and the relative differences in the morphology of the neuronal cells.

Results: Morphological analysis revealed that addition the IL-1β to the culture of isolated DRG neurons supplying porcine urinary bladder did not significantly affect the number of axonal branch points and neurites as well as their length.

Conclusions: This is the first report specifying the share of IL-1β in the regeneration process of the porcine DRG neurons supplying the urinary bladder. A lack of changes in the total axon length, maximal distance of the longest axon and the number of the branch points suggest that this factor has not the capacity to promote regeneration of this specific subpopulation of DRG neurons.

This work was supported by grant no 2011/03/B/NZ4/06451.

|B15|

Influence of nerve growth factor (NGF) on axon outgrowth by dorsal root ganglia (DRG) neurons supplying porcine urinary bladder – an *in vitro* study

Kozlowska A.[1], Mikolajczyk A.[2], Oponowicz A.[1], Majewski M.[1], Klimaschewski L.[3]

[1]Department of Human Physiology, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[2]Department of Public Health, Epidemiology and Microbiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Innsbruck Medical University, Department of Anatomy and Histology, Innsbruck, Austria

It is well know that nerve growth factor (NGF) increases initial rate of axonal regeneration of aged dorsal root ganglia neurons (DRG) and influences on the survival time of these neurons. However, there is a lack of data revealing the putative influence of NGF on the neurite outgrowth of urinary bladder-projecting DRG neurons. Therefore, the present study was aimed at determining the influence of NGF treatment on morphology of the porcine bladder-projecting sensory neurons.

Lumbar and sacral DRGs were harvested from 8-10 weeks old pigs (n = 4) which received multiple injections of the retrograde tracer Fast Blue in the urinary bladder wall 2 weeks prior to the collecting of DRG studied. Cell cultures were prepared as described previously (Hausott *et al*., 2011). Neurons were plated on glass coverslip coated with poly-L-Lysine/laminin and cultivated in TNB medium at 37°C in 5% CO_2 and treated with NGF (100 ng/ml, experimental group). After 24 h in culture, neurons were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.01% Triton X-100 in PBS for 5 min and blocked with blocking buffer (10% goat serum in PBS) for 30 min. Cells were incubated with primary antibodies against neuron-specific β-III tubulin diluted in blocking buffer for 1 h at room temperature and then incubated with secondary Alexa-488-conjugated antibody for 60 min at RT. Neurite length analysis was performed using the WIS-NeuroMath (Rishal *et al*., 2012). The Mann-Whitney *U* test (GraphPad Software, Inc) was used to analyze the variability in the relative differences in the morphology of the neuronal cells.

The result show the number of branch points was increased after NGF treatment, while the total axon length as well as maximal distance of the longest axon was similar to those observed in untreated control.

This is the first scientific paper concerning the influence of NGF on the porcine sensory DRG neurons supplying the urinary bladder during their regeneration. The rise in the number of axonal branch suggests contribution of this factor in the regeneration of axons from DRG neurons.

This work was supported by grant no. 2011/03/B/NZ4/06451.

|B16|

Immunological and Biological Properties of Glial Restricted Progenitors for Potential Application in ALS Therapy

Kozlowska U., Klimczak A., Kurpisz M.

Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland

igcz@man.poznan.pl;
e-mail of presenting author: urszula.kozlowska@igcz.poznan.pl

Introduction: The neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) constitute social problem related to civilization. First results of stem cell therapies in ALS seem to be promising. However, rejection of allogenic neural cells is still a common problem and there is an urgent need to define the mechanism of immune response and to develop effective immunosuppressive strategy. In this study, we evaluated immunogenic potential of glial restricted progenitor cells – the potential candidates for ALS therapy.

Material and methods: Glial Restricted Progenitor cell suspensions (GRP) derived from canine and murine fetuses and obtained human commercial cell line QSV40 were long time cultured in DMEM medium, enriched in growth supplements. The phenotypic characteristics of human, murine and canine GRPs were assessed via flow cytometry and immunofluorescence staining. Cells were evaluated for the presence of immunogenic markers: MHC-I and MHC-II and costimulatory molecules CD40, CD154, CD28 and CD80 as well as for the presence of markers specific for neural progenitors: A2B5, nestin, NG2, and for differentiated cells: β-III-tubulin, PSA-NCAM, GFAP, MBP. Differentiation *in vitro* of GRP cell into astrocytes was performed in culture with 15% FBS media.

Results: GRPs of all examined species express MHC-I but not MHC-II antigens. The presence of costimulatory molecules was not observed on surface of murine and human cells. GRPs preserve stable phenotype of A2B5,

NG2 and nestin in long term culture. Murine and canine GRPs were easily differentiated into astrocytes in medium supplemented with 15% FBS. Such phenomenon was not observed in human QSV40 cell line. Differentiation potential of murine and canine GRP was proven by GFAP expression.

Conclusions: GRPs are stable in long term in vitro culture. There is no expression of MHC-II and costimulatory molecules which might suggest low immunogenic potential of the cells. Not immortalized murine and canine GRPs show ability to differentiate into astrocytes. These biologic properties of GRP make them potential candidates for ALS therapy.

|B17|

Platinum nanoparticles as efficient inhibitors of cells proliferation in glioma

Kutwin M.[1], Sawosz E.[1], Jaworski S.[1], Wierzbicki M.[1], Strojny B.[1], Chwalibog A.[2]

[1]Division of Biotechnology and Biochemistry of Nutrition, Faculty of Animal Science, Warsaw University of Life Science, Warsaw, Poland
[2]Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, Copenhagen, Denmark

Glioblastoma is one of the most frequent primary and aggressive (WHO grade IV) neoplastic malignant tumors of the central nervous system with a poor survival time. Despite developments in neurosurgery and treatment strategy, the chemotherapy is still inefficient and it is urgent to develop new strategies for tumour therapy. The objective of this study was to investigate the effect of platinum nanoparticles (NP-Pt) on the inhibition of glioma cell proliferation and compare the antiproliferative properties of NP-Pt with platinum based drugs – cisplatin against U87 and U118 glioma cell lines and tumour tissue. NP-Pt and cisplatin were incubated with U87 and U118 glioma cells or injected directly into glioma tumour tissue. Herein, we report a study on the antiproliferative properties of NP-Pt by examining the influence of nanoplatinum on the glioma cells morphology, the level of DNA synthesis, viability and cells migration ability of glioma cells, as well as protein expression of proliferating cell nuclear antigen (PCNA) at glioma tumour tissue. The obtained results showed that NP-Pt treatment of U87 and U118 glioma cells caused reduction of DNA synthesis and the migration of cancer cells. Moreover, the PCNA protein expression level was also decreased at tumour tissue after NP-Pt administration. The obtained results demonstrated the antiproliferative properties of NP-Pt. Consequently, NP-Pt can be considered as an effective inhibitor of glioblastoma tumour cell proliferation. However, the molecular mechanism of action as well as potential side effects need to be elucidated in *in vivo* follow-up research.

|B18|

Critical view on the selection criteria of optimal derivation method of Mesenchymal Stem Cells from Wharton's Jelly: phenotypic, functional and safety control

Lech W.[1], Drela K.[2], Zychowicz M.[1], Obtulowicz P.[1], Buzanska L.[1], Domanska-Janik K.[1]

[1]Stem Cell Bioengineering Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Mesenchymal stem cells (MSC) exhibit enormous heterogeneity which can influence their regenerative properties, therapeutic effectiveness and finally their safety after transplantation. The source of stem cells and also the method of isolation, can significantly affect their properties. Human mesenchymal stem cells derived from bone marrow, adipose tissue or umbilical cord, have been used in many clinical trials. Recently Wharton's Jelly-derived MSC (WJ-MSC) gained special attention due to their low immunogenicity, strong immunomodulatory properties and ability to secrete adjuvant factors supporting regeneration. The aim of the study was to characterize and compare WJ-MSC isolated from umbilical cord stroma by mechanical and collagenase based enzymatic process and critical view on the selection criteria of optimal MSC derivation method.

Both obtained cell populations were characterized followed by ISCT criteria (expression of typical surface markers, mesodermal differentiation potential) and additionally a number of such features for MSC as proliferation and senescence rate, self-renewal capacity based on ability to create specific colonies (CFU-F) and neural differentiation potential. Cells were analyzed with flow cytometry, immunocytochemistry and quantitative RT-PCR techniques.

Despite comparable level of expression of typical for MSC markers, enzymatically isolated cells were less stable in culture with slower proliferation rate, low frequency of CFU-F formation ability, faster cell senescence and limited

mesodermal differentiation potential. Moreover a significantly higher expression of neuronal and glial markers: Nestin, βTubulinIII, GFAP, NF-200 as well as primitive marker α-SMA was observed in WJ-MSC obtained by mechanical tissue fragmentation. These unique properties of WJ-MSC for neural differentiation can lead to their promising future applications in the treatment of neurological disorders.

To guarantee the high quality of the isolated cells, we should focus on necessity of development of more sensitive and selective methods for prediction and control cells function during time of growth *in vitro*.

The work was supported by National Science Center grant no. 05401/B/NZ3/2011/01 and National Centre for Research and Development grant No STRATEGMED1/234261/2/NCBR/2014.

|B19|

Measuring movement of stem cells placed in electric field – methods and problems

Lipinski S.[1], Jezierska-Wozniak K.[2,3], Wojtkiewicz J.[3,4], Habich A.[2,3], Grabarczyk L.[2], Barczewska M.[2], Maksymowicz W.[2,3]

[1]Department of Electrical Engineering, Power Engineering, Electronics and Automation, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[2]Department of Neurology and Neurosurgery, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Laboratory for Regenerative Medicine, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[4]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
seweryn.lipinski@uwm.edu.pl

One of the often-appearing problems in case of use of stem cells in regenerative medicine is evaluation of their movement when they are placed in electric field. That issue can be considered in two ways. On the one hand, desirable can be directed migration of stem cells. That is the case of the idea of delivering stem cells as close to their destination as it is possible, and then guiding their migration using electric field. On the other hand, desirable can be as well reduction of cells movement, i.e. keeping them in the place of delivery, also using electric field. In both cases, the key is selection of proper kind and strength of electric field. Research on this matter is now quite common. However, in order to study the influence of electric field on stem cells movement, necessary are reliable methods provided for precise determination of cells position and its changes over time. The aim of our study was to test whether different methods used for these purposes can influence on the evaluation of research results. We based our study on series of images obtained from digital microscope. Adult human bone marrow-derived mesenchymal stem cells were being placed in pulsed electric fields of different strengths and frequencies. In each case, images were acquired every 15 minutes, for duration of 3 hours. Then, locations of the individual cells, as well as parameters of their movement (i.e. velocity and linearity) were estimated using different methods. The results show that the influence of used method is not to be underestimated as it can have a significant impact on the evaluation of research results. Problems concerning different methods of evaluation of the migration of stem cells in electric field were listed as well, including their comparison in aspects of implementation and use.

|B20|

Magnetic resonance imaging in the evaluation of the changes in intervertebral discs under treatment – methods and problems

Lipinski S.[1], Jezierska-Wozniak K.[2,3], Wojtkiewicz J.[3,4], Habich A.[2,3], Barczewska M.[2], Maksymowicz W.[2,3]

[1]Department of Electrical Engineering, Power Engineering, Electronics and Automation, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[2]Department of Neurology and Neurosurgery, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[3]Laboratory for Regenerative Medicine, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
[4]Department of Pathophysiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland
seweryn.lipinski@uwm.edu.pl

Diseases of the intervertebral discs (discopathies) are one of the most common diseases of civilization. Discopathy is initiated through biochemical changes in the structure of discs. In this context, tissue engineering, through creating new possibilities of regeneration of degraded tissue, raises hope that there appear new and more effective methods of dealing with this problem. To evaluate the effectiveness of therapies most often are used

animal models in which in a certain way the process of degeneration of the intervertebral disc is initiated. Most commonly used method of determination of the degree of damage and possible subsequent regeneration of intervertebral discs is magnetic resonance imaging (MRI). Our study focused on the identification of differences that may arise in the assessment of the same cases based on images obtained in different cross-sections of the same MRI research. The issue of proper indication of area of interest has been raised as well, which is, as shown by clinical experience, especially problematic after a long period (i.e. several months) from the time of injury. Attempt was made to identify the most reliable methodology, which could be used to assess the degree of degeneration/regeneration of intervertebral discs. Images obtained in MRI study were analyzed to evaluate the process of regeneration of intervertebral discs after administration of mesenchymal stem cells isolated from bone marrow in porcine animal model, after the damage caused by the surgical laser vaporization. Evaluation was performed for the time of 4, 8 and 12 weeks from the time of damage. Based on our study, the following conclusions can be drawn. Firstly, due to the greater number of available measurement data, frontal plane can be indicated as more reliable than sagittal. Secondly, use of the one of the basic geometrical shapes as the discs' shape approximation can lead to significant errors in the evaluation of the results of the experiment – the borders of the intervertebral disc should be determined using more complex shapes, i.e. considering the anatomy.

|B21|

Influence on the cell body of neurons in central nerve system of cytokines by condition of demyelination

Melnyk N.[1], Labunets I.[2], Rymar S.[2]

[1]Histology and Embriology Department, O. O. Bogomoletz National Medical University, Cell and Tissue Technologies Department, Gene Technologies Department, Institute of Genetic and Regenerative Medicine NAMS of Ukraine, Kiev
[2]Cell and Tissue Technologies Department, Gene Technologies Department, Institute of Genetic and Regenerative Medicine NAMS of Ukraine, Kiev

To assess the relationship of morphological status of the neurons of the cerebrum, cerebellum and spinal cord and behaviour of mice with the "cuprizone" model of demyelination and remyelination after used of exogenous recombinant human leukemia inhibitory factor and interleukin 10. Adult mouse of line 129/Sv received daily for three weeks "cuprizone" with food. Control mice received normal food. After first week of cuprizone application mice were provided with exogenous recombinant human leukemia inhibitory factor and interleukin 10. The animals were evaluated for morphometric analysis which determined the proportion of neurons with unmodified, and with moderate and severe structural changes (staining of histological specimens of toluidine blue) and behavioral reactions (open field test). In morphological investigations we observed structurally modified neurons in the gray matter of the cerebrum, cerebellum and the spinal cord of all experimental groups of mice line 129/Sv. "Cuprizone" had a stronger psychological impact to most behavioral responses in mice. After use of exogenous recombinant human leukemia inhibitory factor and interleukin 10 we observed an increase in unmodified neurons in all parts of the central nerve system. The severity of violations of behavioral and pathological changes of neurons in the CNS after taking "cuprizone" and cytokines are harmonized and have clear differences. The experimental model using "cuprizone" aplication can be used as a model of the demyelination neurological degeneration and breaches of conduct.

|B22|

G1222A polymorphism of HCRTR1 gene and the concentration of hypocretin-1 in patients with migraine

Mrzewka B.[1,2], Kowalska M.[1], Kapelusiak-Pielok M.[3], Kozubski W.[3], Dorszewska J.[1]

[1]Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland
[2]Students Scientific Neurobiological Association, Poznan University of Medical Sciences, Poznan, Poland
[3]Chair and Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland

Hypocretin-1 is a hypothalamic neuropeptide with a known chemical structure and anatomical distribution. This peptide is a part of the hypocretin system which is linked with central pain modulation, probably by way of influence on trigeminovascular nociception and the cortical spreading depression. Recent genetic studies supports the position that hypocretins may play a role in the pathogenesis of primary headache disorders and the hypocretin receptor 1 (HCRTR1) gene could contribute to susceptibility to migraine without aura. The objective of this paper is to investigate the association between non-synonymous G1222A polymorphism of the HCRTR1 gene and

the concentration of hypocretin-1 in 96 migraine patients (11 males, 85 females) representing different subtypes of the disease: migraine with aura (MA: 43 patients) and migraine without aura (M0: 53 patients). A group of 82 healthy controls were subsumed under the study (10 males, 72 females). Polymorphism in exon 7 of HCRTR1 gene results in an amino acid substitution in the cytoplasmic region of the HCRT receptor and could modify its function. Polymorphism G1222A is associated with an increased risk of disease, probably due to the carriage of the A allele of HCRTR1 gene. The results of the study revealed significant difference in the frequencies of the GG, GA, and AA genotypes between patients with migraine as a whole and with M0 when compared with control subjects ($p < 0.05$, Fisher's exact test). The prevalence of genotypes did not reached statistically considerable difference in patients with MA. Interestingly enough, significant differences in the serum concentration of hypocretin-1 were observed among AA individuals. However, the research did not demonstrate diversity of the blood serum concentration of hypocretin-1 in migraineurs (648.1 ± 343.7 pg/ml) to compare with controls (670.3 ± 406.0 pg/ml; $p = 0.4610$) as well as when the patients were divided into migraine with and without aura groups ($p = 0.8655$). In connection with the above-mentioned facts it could be assumed that described genotypes did not seem to modify the serum concentration of hypocretin-1, except for AA genotype, which is associated with an increased migraine risk. The study revealed no relationship between hipocretin-1 and subtypes of migraine.

|B23|

Vasculo- and neuroprotective ability of mesenchymal stem cells derived from Wharton jelly in postischemic rat brains

Obtulowicz P., Lech W., Zychowicz M., Sarnowska A., Domanska-Janik K.

Stem Cell Bioengineering Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Mesenchymal stem cells derived from Wharton jelly (WJ-MSC) appear to be promising candidate for postischemic tissue repair. Unfortunately, moderate and inconsistent benefits of cellular clinical trials press for an urgent need to improve this therapy. The aim of our study was to evaluate the ability of WJ-MSC to differentiate into endothelial progenitor cells (WJ-EPC) and compare the influence of these both cell types on vascular network and proliferation of neural cells in ischemically injured rat brain. WJ-MSC were cultured in growth medium (MSCGM) or in endothelial differentiating medium (EGM-2). Cells were characterized by flow cytometry, immunocytochemistry and molecular methods on the basis of expression of endothelial and mesenchymal markers. Angiogenic activity of WJ-EPC was proved by DiI-Ac-LDL-uptake and Matrigel assay. Cells metabolic activities were determined by WST-1 reagent. Cytotoxic brain injury was conducted with the use of Na^+/K^+ pump inhibitor – ouabain (1 µl; 50 nmol). WJ-MSC and WJ-EPC were engrafted in 3D bioactive platelet lysate (PL) scaffolds into the striatum of normal and focally injured brains of adult Wistar rats. After 3, 7, 14 and 21 days brains were isolated and sliced with cryostat. Subsequently, immunohistochemical studies was performed with rat endothelial cell specific antibody (RECA-1), cell proliferation marker (Ki67), neural markers (Nestin, NF-200, β-tubulin III, GFAP) and microglial cell marker (ED1). WJ-MSC after 7 days in EGM-2 medium, acquired typical EPC cobblestone-like morphology, form capillary-like structures on Matrigel and took up DiI-Ac-LDL. Both cell types were positive for MSC and EC markers CD73, CD90, CD105, VEGFR2, VEGF, but only EPC culture expressed vWF and CD31 on significantly higher gene and protein levels. We showed that plateled lysate could be transplanted into the brain in rat models. Scaffolds with WJ-MSC and WJ-EPC exert vasculo- and neuroprotective properties after transplantation into striatum of focally injured rat brains which makes them a good candidate for cell therapy.

The work was supported by NCN grant 2011/01/B/NZ3/05401 and NCRD grant Strategmed 1/234261/2/NCBR/2014.

|B24|

The influence of the dosage drug form on increasing the effectiveness of the therapy of neurodegenerative diseases

Ostrozka-Cieslik A., Sarecka-Hujar B., Jankowski A.

Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Department of Pharmaceutical Technology, Chair of Applied Pharmacy, Sosnowiec, Poland

beatasarecka@poczta.onet.pl

Background: In recent years, the dynamic process of aging of the population is observed. It is estimated that

in 2025 the number of people aged over 60 will exceed the threshold of 1 billion, and the proportion of elderly will reach almost 14% of the world population. The number of people suffering from neurodegenerative diseases is also increasing. Effective treatment of these diseases is very important, and at the same time difficult. Currently, numerous studies focusing on finding effective, safe and simultaneously minimally invasive methods of drug therapy are conducted. The development of drug form technology allows to optimize the currently used treatment regimens and develop the new ones. The aim of the study was to analyze current data concerning drug forms used in the treatment of neurodegenerative disorders.

Material and methods: An analysis of the available literature of the past 16 years was conducted. We searched the following databases: PubMed, Medline, Embase, the Cochrane Library as well as the scientific medical journals.

Results: Recently, besides the traditional dosage forms like tablets and capsules, sustained release tablet, disintegrating tablets in the oral cavity, and transdermal patches were allowed in the treatment of Alzheimer's and Parkinson's diseases. Sustained release tablets contain increased amounts of the drug substance that provides the constant effective concentration of at least 8-12 h. The tablets disintegrating in the mouth are the safe drug for people with the swallowing problems thus convenient for elder patients. They disperse on the tongue, have a rapid onset of action of the active substance and minimize first-pass effect. The transdermal systems have the form of a patch, that allows administering the drug substance to the general circulation. Several nanoformulations containing the drug (e.g. nanoemulsions, lipid nanoparticles, polymeric micelles) which enable the targeted therapy, increase its efficacy and bioavailability of the drug are also tested. The nanoparticles have the ability to penetrate the blood-brain barrier and demonstrate biodegradability, stability and low toxicity.

Conclusions: The development of nanotechnology of drug formulation increases the chances of an effective and safe treatment of neurodegenerative diseases.

|B25|

Exogenous cholesterol and non-sterol isoprenoids stimulate clusterin protein levels in PC-12 cells with APP overexpression

Pajak B.[1,2], Kania E.[1], Orzechowski A.[1,2]

[1]Electron Microscopy Platform, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW, Warsaw, Poland

orzechowski_arkadiusz@wp.pl

Previously, we reported that PC-12 cells overexpressing wild type APP(-wt), or Swedish mutation APP(-saw) under control of GFP gene promoter have reduced viability escorted by enhanced autophagy. Present study was conducted to address the question whether mevalonate pathway affects the level of clusterin protein, the extracellular chaperone that protects cell viability. Clones stably expressing wild type APP (W), or Swedish mutation APP (S) under control of GFP gene promoter together with empty vector (G) transfected cells were differentiated into neuronal cells with NGF. APP overexpression was monitored by the presence of green fluorescence (FL) and Western blot (WB). FL confirmed stable transfection whereas WB showed sAPPalpha fragment present in APP(-wt) but not in APP(-sw) or empty vector transfected cells. Similarly, amyloid beta (Abeta) was found solely in APP(-sw) but not in APP(-wt) or empty vector transfected cells. Next, the cells were left untreated or treated with selected statins or non-sterol isoprenoids or both for 24 h. Atorvastatin [ATR, 50 microM] or simvastatin [SIM, 50 microM] used to inhibit mevalonate synthesis (HMG-CoA reductase inhibitors) diminished cell viability ($p < 0.05$) but did not affect clusterin protein levels. Interestingly, administration of non-sterol isoprenoids (geranyl-geraniol – GGOH, farnesol – FOH, mevalonate – MEV) and cholesterol PEG conjugate (Chol-PEG) in non-toxic concentrations strongly stimulated ($p < 0.05$) clusterin protein levels in PC-12 cells. The effects of Chol-PEG and non-sterol isoprenoids were more profound in APP(-wt) and APP(-sw) than in empty vector transfected cells. GGOH overridden statin-dependent cytotoxicity by re-establishing cell viability to observed in untreated cells but this effect does not seem to be dependent on clusterin as its level did not differ from other treatments. These results suggest important role played by mevalonate pathway in clusterin protein stimulation in APP overexpressing cells, although merely GGOH has direct effect on cell survival. Protein prenylation with GGOH

rather than FOH might play substantial role in neuronal cell viability.

This research was funded by National Science Centre (Poland), Grant no. UMO-2011/03/B.

|B26|

Stemness or differentiation? The influence of small molecules and oxygen tension on neural stem cells fate decisions

Podobinska M., Winiarska H., Buzanska L.

Stem Cell Bioengineering Unit, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Small molecules can exert developmental context dependent activity: influence the maintenance of stem cells stemness or support their differentiation into specific cell types. Moreover, it was proven that oxygen tension may influence the cellular response to small molecules. In this study the effect of selected small molecules (TSA, RG108, PD0325901, SB431542) and the oxygen tension on developmental processes of neural stem cells derived from human umbilical cord blood (HUCB-NSC) was investigated. We have shown that 5% oxygen concentration promotes proliferation of HUCB-NSCs, but the presence of selected small molecules affect this correlation. HUCB-NSC cultured in 21% oxygen concentration with RG 108 revealed significantly higher proliferative potential.

All samples have been tested for the expression of genes typical for the pluripotency (OCT4, SOX2, NANOG, REX1) neural differentiation (B-TUBULIN III, MAP2, NF200) as well as related to the hypoxia (HIF1, 2, and 3 alpha) and epigenetic regulation (HDAC1, HDAC2, DNMT3A, DNMT3B). This expression was confirmed on the protein level by immunocytochemistry. In all tested experimental variants cells were negative for OCT4, SOX2 and REX1. Low NANOG expression was detected in two variants: HUCB-NSCs cultured in 5% oxygen concentration without addition of any small molecule and with TSA. Neural differentiation of HUCB-NSC was confirmed by the expression of neural markers in all tested samples, with two exceptions: there was no expression of MAP2 in cells cultured in 5% oxygen concentration with RG108 and NF200 in cells growing in 21% oxygen concentration with TSA. The expression of genes involved in regulation of epigenetic processes (HDAC1, HDAC2, DNMT3A, DNMT3B) was present in HUCB-NSCs in almost all experimental conditions, except cells growing in 21% oxygen with PD0325901 – for all tested genes and cells cultured in 5% oxygen concentration with RG108 – for DNMT3A, DNMT3B exclusively. The quantitative estimation of the expression of selected genes is presently under verification.

Our study based on changes of the expression of investigated genes revealed, that developmental response of HUCB-NSCs to small molecules may be modified by the level of oxygen tension in cell microenvironment.

The work was supported by MMRC statutory funds.

|B27|

Association between 1298A>C polymorphism within MTHFR gene and paediatric ischemic stroke – meta-analysis of 325 cases and 504 controls

Sarecka-Hujar B.[1], Kopyta I.[2], Ostrozka-Cieslik A.[1], Sordyl J.[3]

[1]Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Department of Drug From Technology, Sosnowiec, Poland
[2]Medical University of Silesia in Katowice, School of Medicine in Katowice, Department of Paediatrics and Developmental Age Neurology, Katowice, Poland
[3]Medical University of Silesia in Katowice, School of Medicine in Katowice, Department of Paediatrics and Paediatric Endocrinology, Katowice, Poland

beatasarecka@poczta.onet.pl

Background: It is observed that the level of homocysteine (HCys) plays important role in the functioning of endothelial cells. Elevated level of HCys is a risk factor for vascular diseases as well as brain atrophy. Several common polymorphisms within genes encoding enzymes involved in homocysteine metabolism (e.g. methylenetetrahydrofolate reductase; MTHFR) are suggested to be associated with development of arterial ischemic stroke (AIS), both in adults and children. Previous studies, including meta-analysis, have shown that 677C>T polymorphism within MTHFR gene is related to paediatric AIS. However, the role of other common MTHFR polymorphism, 1298A>C, is still uncertain. One studies show such relation, while others – do not. Most often, studies are performed on small number of patients due to the rarity of AIS. Considering that, we performed meta-analysis of available data addressing possible association between MTHFR 1298A>C polymorphism and AIS in children.

Material and methods: We searched Pubmed using "MTHFR polymorphism", "ischemic stroke", "paediatric", "1298A>C polymorphism", "children" as keywords. We included to a study 5 case-control studies. Due to the possibility of obtaining false positive or negative results, we did not include to the present meta-analysis studies with the number of patients lower than 40. A total number of 325 paediatric patients with arterial ischemic stroke and 504 controls were enrolled to the study. We conducted statistical analyses with the use of MedCalc software. Heterogeneity between the studies was evaluated using the Dersimonian and Laird's Q test. When heterogeneity between the studies was significant, the pooled odds ratio (OR) was analysed with a random effects model, otherwise, a fixed effects model was used. Results: In case of MTHFR AC+CC vs AA analysis, we observed significant heterogeneity between the analysed studies (Cochrane Q $p = 0.02$ and I2 = 65.19%), thus random effects model was used to estimate pooled OR. We observed that carrier-state of 1298C allele is not related to ischemic stroke in children ($p = 0.236$, OR = 1.37, 95% CI: 0.81-2.30).

Conclusions: The results based on a sizeable groups of paediatric patients suffering AIS and healthy controls demonstrated that 1298A>C polymorphism in MTHFR gene is not related to paediatric ischemic stroke.

|B28|

Neurochemical and neuropathological aspects of new psychoactive substances ("legal highs") use

Skowronek R.[1], Kobek M.[1], Pieprzyca E.[1], Palasz A.[2]

[1]Department of Forensic Medicine and Forensic Toxicology, School of Medicine in Katowice, Medical University of Silesia in Katowice, Katowice, Poland
[2]Department of Histology, Chair of Histology and Embryology, School of Medicine in Katowice, Medical University of Silesia in Katowice, Katowice, Poland

The presence of new psychoactive substances (NPS) use is a relatively new challenge for clinicians, forensic pathologists, lawyers and basic researchers. According to alerts of European Union Early Warning System (EU EWS), almost each week completely new substance (mainly synthetic cannabinoids, cathinones and opioids) appears on drugs of abuse market. These substances are often called "legal highs" due to the fact that after introduction on the narcotic market they are not illegal. The main current problem is lack of sufficient knowledge about its mechanism of action, psychoactive effects in humans and its acute, short- and long-term toxicity.

The aim of this work is: (1) to review the published and referenced in PubMed database results of basic studies on the mechanisms of action and toxicity of some of NPS, and (2) present own results of routine neurohistological examination (H&E stain) of the central nervous system (CNS) in fatal victims of NPS intake, examined post mortem in the Department of Forensic Medicine and Forensic Toxicology in Katowice on the prosecutor's order.

New designer drugs mimic the action of classical narcotics. In the last years the number of articles concerning mechanisms of action and toxicity of some of NPS is constantly growing, but still relatively small number of NPS was studied. There are many questions, which have to be answered. Our results of routine neurohistological examination of CNS in fatal victims of NPS intake indicate that there are no specific changes related with NPS intoxication. In all cases we found severe oedema and hyperaemia. Further studies on animal models (*in vitro*, *in vivo*) and on human post mortem material obtained during autopsy are strongly required for better understanding of NPS action, which is important for both, clinicians and forensic experts.

|B29|

Activation of NG2-positive glia after transient cerebral ischemia in the rat – immunocytochemical studies

Steliga A.[1], Waskow M.[1], Lietzau G.[2], Wojcik S.[2], Morys J.[2], Kowianski P.[1,2]

[1]Department of Health Sciences, Pomeranian University in Slupsk, Slupsk, Poland
[2]Department of Anatomy and Neurobiology, Medical University of Gdansk, Gdansk, Poland

Reactive gliosis has been regarded as the specific and stimulus-dependent character of nervous tissue response to different pathological processes. Important issues for this reaction are cellular proliferation and de-differentiation of resident progenitors enabling their further development into various cell lineages. The source of differentiating cells and the intensity of their proliferation in different pathological circumstances are still the subject of discussion. A significant role is attributed to NG2-immunoreactive (NG2-ir) polidendrocytes (oligodendroglial progenitor cells), which constitute the most numerous population of progenitors in the developed brain. The aim of this study

was to assess the proliferation and differentiation capabilities of NG2 glia during reperfusion after 1 h transient cerebral ischemia in rats. An ischemia was evoked in adult male Wistar rats with subsequent 6 weeks of reperfusion in order to assess the reactive glial response. Transient cerebral ischemia triggers an intense proliferative reaction of polidendrocytes and astroglia. However, our study did not reveal the ability of changing the morphological features of glial lineage fate among the former and replenishing the population of reactive astrocytes.

|B30|

Alteration in gene expression of sphingolipid metabolism enzymes in PC12 cell line transfected with amyloid precursor protein

Wencel P.L., Strosznajder R.P.

Laboratory of Preclinical Research and Environmental Agents, Department of Neurosurgery, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

pwencel@imdik.pan.pl

Background: Sphingolipids are diverse class of lipids that play an important role in signal transduction, cell recognition, growth arrest and apoptosis. These lipids include sphingomyelin, ceramides, ceramide phosphate, sphingosine 1-phosphate (S1P). Disturbances in sphingolipid metabolism and alteration of the rheostat between ceramide and S1P have been suggested as important factors in pathogenesis/pathomechanism of Alzheimer's disease (AD). This study concentrate on the effect of endogenously liberated amyloid beta peptides (AB) on enzymes' gene expression involved in sphingolipid metabolism in pheochromocytoma (PC12) cells characterized by overproduction of amyloid precursor protein (APP).

Methodology: Rat PC12 cells transfected with human gene for wild type of APP (APPwt) and PC12 cells bearing double Swedish mutation (APPsw) were used in this study. Control PC12 cells were transfected with an empty vector. Biochemical and qPCR methods were applied. Following genes were analyzed: ceramide synthases: Cers2, Cers3; ceramidase: alkaline ceramidase Acer1; kinases: ceramide kinase Cerk1, sphingosine kinases: Sphk1, Sphk2.

Results: Our results demonstrated that endogenously liberated AB (APPwt and APPsw) significantly affected transcription of enzymes involved in the regulation of ceramide level in cells. Analysis of mRNA level for ceramide synthases and ceramidases in both cell lines demonstrated that Cers3 gene expression was upregulated in APPwt cells while expression of Cers2 remained unchanged. However, in APPsw cells significant reduction of Cers2 gene expression was observed. Moreover, the expression of Acer1 gene was decreased in APPwt and APPsw cells. Our study indicated downregulation in Cerk1 gene expression in APPsw cells. Additionally, significant reduction of the gene expression of Sphk2, was found in both APP transfected cells.

Conclusions: Our data indicated significant alterations in enzymes gene expression involved in regulation of balance between bioactive lipids: ceramide and S1P in cellular AD model. The observed changes of Cers, Acer1, Cerk1 and Sphk2 may lead to higher ceramide level, cells' degeneration and death. All these enzymes could be considered as a promising target(s) for cytoprotection against AB toxicity.

Supported by the National Science Centre (http://www.ncn.gov.pl) grant no. NCN/15/B/NZ3/01049.

|B31|

Poly(ADP-ribose) polymerase-1 as a crucial regulator of nuclear and mitochondrial Sirtuins transcription. Implications for cell physiology and pathology

Wencel P.L.[1], Strosznajder J.B.[2], Strosznajder R.P.[1]

[1]Laboratory of Preclinical Research and Environmental Agents, Department of Neurosurgery, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
[2]Department of Cellular Signalling, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

pwencel@imdik.pan.pl

Background: Poly(ADP-ribose) polymerase-1 (PARP-1) is the oldest member of NAD+ dependent PARPs family. In the brain PARP-1 is responsible for more than 90% of protein poly(ADP-ribosylation). PARP-1 is involved in DNA repair, however its overactivation may lead to cell death. The interaction between PARPs and other NAD+ dependent enzymes – histone deacetylases class III - Sirtuins (SIRTs) is fundamental for cells metabolism, DNA repair, transcription in physiology and pathology.

Aim: This study was focused on the role of PARP-1 in regulation of SIRTs gene expression in PC12 cells under resting conditions and amyloid beta (AB) toxicity. The short term effect of AB42 was compared with long term

action of endogenously liberated AB in PC12 cells transfected with Amyloid Precursor Protein (APP).

Methodology: The experiments were carried out using PC12 cells treated with PARP-1 inhibitor PJ-34 (20uM) or with AB42 oligomers (ABO, 1uM) during 24 h in culture. Rat pheochromocytoma (PC12) cells transfected with human gene for wild type of APP (APPwt) and PC12 cells bearing double Swedish mutation (APPsw) were used in this study. Control PC12 cells were transfected with an empty vector. Biochemical and qPCR methods were applied.

Results: Our results demonstrated that pharmacological inhibition of PARP-1 activity by PJ-34 upregulated expression of both nuclear Sirtuins: Sirt1 and Sirt6 in resting conditions and in the presence of ABO. Inhibition of PARP-1 had no effect on cytosolic Sirt2 expression and its signalling. However, PJ-34 enhanced gene expression for mitochondrial Sirt4 which is mainly responsible for mono(ADP-ribosylation) and indicates very low deacetylase activity. ABOs upregulated mRNA level for both mitochondrial Sirtuins: Sirt4 and Sirt5 and PJ-34 decreased their expressions. Endogenously liberated AB peptides enhanced mRNA level exclusively for mitochondrial Sirt3 in APPsw cells suggesting its involvement in antioxidative defense.

Conclusions: Summarizing, these results indicate that PARP-1 is a very important regulator of Sirtuins transcription in control resting condition and in AB toxicity. Mutual dependence between PARPs and SIRTs can influence nuclear and mitochondrial protein functions and cells metabolism in physiology and in AB toxicity.

Supported by The National Science Centre (http://www.ncn.gov.pl) Grant 2013/09/B/NZ3/01350

|B32|

Breast-milk-derived cells – connection with postnatal development of nervous system?

Witkowska-Zimny M., Mikulska A., Wrobel E.

Department of Biophysics and Human Physiology, Medical University of Warsaw, Warsaw, Poland

mwitkowska@wum.edu.pl

Introduction: Breast-feeding plays an important role for the development of the newborn. The breast milk among proteins, lipids, carbohydrates and other biologically active components also contains a heterogeneous population of cells that have the potential to differentiate into various mature cell types including neural cells [1]. The natural presence in breastmilk of stem cells with multilineage differentiation potential raises the question of the role of these cells during early infant development. It is proposed that the cells could develop the enteric nervous system – one of the main divisions of the nervous system, consists of a mesh-like system of neurons, that governs the function of the gastrointestinal system. Non-breast fed premature born infants show a significantly higher risk of developing diseases like infantile diarrhoea and necrotizing enterocolitis.

Material and methods: In aseptic procedures, mature breast milk (10-30 ml) samples were obtained from healthy breastfeeding women with early range of lactation stages. The isolation procedure was based on the protocols described by Hassiotou *et al*. [2] with modifications. The cells were cultured under standard conditions in basic culture medium based on low-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic–antimycotic mixture.

Results: The aims of this study were to obtain the breast milk-derived cells cultured in vitro. The isolated breast-milk-derived cells were adherent to the plates. The presence of the cells with various origins was detected in human breast milk.

Conclusions: Human breast milk contains a heterogeneous population of cells that have the potential to provide a non invasive source of cells for proper growth and development of infants, but also for cell therapy and treating neonatal disorders. Because of its ethical, noninvasive and plentiful nature, breastmilk offers a novel resource of patient-specific progenitor cells for applications in regenerative medicine and also in treating neurodegenerative diseases.

Acknowledgment: We thank dr Elżbieta Łodykowska for collecting milk samples.

References

1. Hosseini S.M. et al. Differentiation of human breast-milk stem cells to neural stem cells and neurons. Neurol Res Int. 2014, 201.
2. Hassiotou F. et al. Breastmilk is a novel source of stem cells with multilineage differentiation potential. Stem Cells. 2012, 2164-2174.

|B33|

Upconverting Nanoparticles: a new approach for biomedical applications. Cytotoxicity and uptake mechanism of the NaYF4:Yb3+,Er3+ nanoparticles.

Zajdel K.[1], Sikora B.[2], Fedoryszak-Kuska N.[3], Kowalik P.[2], Fronc K.[2], Kaminska I.[2], Szewczyk M.[5], Longo J.P.[6], Stepien P.P.[3,4,5], Elbaum D.[2], Frontczak-Baniewicz M.[1]

[1]Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland
[2]Institute of Physics, Polish Academy of Sciences, Warsaw, Poland
[3]Centre of New Technologies, University of Warsaw, Warsaw, Poland
[4]Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
[5]Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland
[6]Instituto de Ciências Biológicas, Departamento de Genética e Morfologia, Universidade de Brasilia, Brasilia DF 70919-970, Brazil

A new generation of multifunctional nanoparticles, defined as upconversion nanoparticles (UCNPs), are luminescent nanomaterials, which have ability to convert near infrared light (λ = 980 nm) to visible or UV light. The unique properties of the UCNPs are: low toxicity and low autofluorescence from biological samples. They are very useful candidates for in vitro cellular imaging and in vivo tissue imaging. The UCNPs have also potential applications in photodynamic therapy, drug delivery, biomolecules sensing and theranostics. To understand biological interactions between nanoparticles (NPs) and living cells, at the molecular level, we studied their cellular internalization process termed endocytosis. The endocytosis takes several forms: clathrin-mediated endocytosis (CME) and clathrin-independent pathways (CIE) including lipid-raft/caveolin-mediated endocytosis, micropinocytosis and phagocytosis. To examine which mechanism is involved in the NPs endocytosis we applied several inhibitors. Chlorpromazine was used to block clathrin-mediated endocytosis. Methyl-β-cyclodextrin (MβCD) and nystatin were evaluated as inhibitors of caveolae-mediated endocytosis; amiloride and cytochalasin D were used to inhibit macropinocytosis and phagocytosis, respectively. Sodium azide and low temperature (4°C), which are factors inhibiting almost all endocytic pathways were used to distinguish from the non-endocytic pathways. Influence of the β-NaYF4:Yb3+,Er3+ upconverting nanoparticles on cell viability and proliferation was evaluated by the following assays: MTT, PrestoBlue and RealTime MT-Glo Cell Viability Assay. Effects of endocytosis inhibitors on the internalization of β-NaYF4:Yb3+,Er3+ UCNPs by HeLa and HEK293 cells were evaluated using confocal microscopy and transmission electron microscopy (TEM). Pre-treatment of HeLa and HEK293 cells with the tested endocytosis inhibitors showed that more than one mechanism is engaged in the cellular uptake of the NPs. The NPs uptake was very fast. We found that the β-NaYF4:Yb3+,Er3+ nanoparticles entered cells as soon as 1 hour post transfection and colocalized with late endosomes and lysosomes. We did not observe ultrastructural changes and the level of apoptosis did not increase in comparison with the control group. The Energy-dispersive X-ray analysis (EDX) confirmed the presence of the UCNPs inside cells and their chemical content. The β-NaYF4:Yb3+,Er3+ UCNPs are promising agents in biomedical science because they revealed no apparent cytotoxicity and are very interesting for multi-functional optical imaging and therapy.

|B34|

Beneficial role of MMPs in neurogenesis after Sodium Butyrate treatment in a model of neonatal hypoxia-ischemia

Ziemka-Nalecz M., Gajos M., Jaworska J., Sypecka J., Zalewska T.

NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

mnalecz@imdik.pan.pl

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix and carry out key functions during brain development but are also implicated in pathological functions within central nervous system. It was reported that histone deacetylase inhibitors (HDACis) provide neuroprotection against brain injury in adult stroke models, among others, by reduction of MMPs activity. Aim of our study was to investigate the influence of HDACi-Sodium Butyrate (SB) treatment on the activity of MMPs as well as its potential role in neurogenesis in the immature brain subjected to hypoxia-ischemia. We utilized a model of hypoxia-ischemia (HI) induced in 7-days old rats. After ligation of the left common carotid artery animals were exposed to hypoxia (7.6% oxygen for 60 min). Hypoxic undamaged hemisphere and sham-operated rats served as control. SB (300 mg/kg) was injected subcutaneously for 5 days starting immediately after HI. The activity of MMPs was examined using *in-situ* zymography. Hypoxia-ischemia resulted in elevation of MMPs activity mainly in hippocampus of the ipsilateral hemisphere

and SB treatment significantly decreased this activation. To investigate the role of MMPs in neurogenesis after SB treatment we used double immunohistochemical staining with specific markers. The administration of SB stimulates the generation of neuroblasts and progenitors of oligodendrocytes and elevates MMPs activity in these cells. These results suggest that SB treatment has beneficial effects by diminishing MMPs activity in acute phase of HI damage and by stimulating the proliferation of neural progenitors and MMPs activation in later stages after injury.

Supported by National Science Centre grants: 2012/05/B/NZ3/00436 and 2015/17/N/NZ7/00969.

Instructions to Authors

This instruction is based upon *Uniform Requirements for Manuscripts Submitted to Biomedical Reviews* (the complete document appears in N Engl J Med 1997; 336, 309-315).

Aims and scope

Folia Neuropathologica is an official journal of the Mossakowski Medical Research Centre Polish Academy of Sciences and the Polish Association of Neuropathologists. The journal publishes original articles and reviews that deal with all aspects of clinical and experimental neuropathology and related fields of neuroscience research. The scope of journal includes surgical and experimental pathomorphology, ultrastructure, immunohistochemistry, biochemistry and molecular biology of the nervous tissue. Papers on surgical neuropathology and neuroimaging are also welcome. The reports in other fields relevant to the understanding of human neuropathology might be considered.

Publication charge

Please note that there is an obligatory charge (250 Euro) for the manuscript being accepted for publication in *Folia Neuropathologica*. We send invoice for payment after the article is accepted for publication. There are no additional charges based on color figures or other elements.

Ethical consideration

Papers describing animal experiments can be accepted for publication only if the experiment conforms to the legal requirements in Poland as well as with the European Communities Council Directive of November 24, 1986 or the National Institute of Health Guide (National Institute of Health Publications No. 80-23, Revised 1978) for the care and use of Laboratory Animals for experimental procedure. Authors must provide a full description of their anesthetics and surgical procedures. Papers describing experiments on human subjects must include a statement that experiments were performed with the understanding and consent of each subject, with the approval of the appropriate local ethics committee.

Submission of manuscripts

Articles should be written in English. **All new manuscripts should be submitted through the online submission at http://panel2.termedia.pl/fn**
For authors unable to submit their manuscript online, please contact with Prof. E. Matyja, Editor-in-Chief of *Folia Neuropathologica*, ematyja@imdik.pan.pl
The Editorial Board reserves the right to reject a paper without reviewers' opinion if the content or the form of the paper does not meet minimum acceptance criteria or if the subject of the paper is beyond the aims and scope of the journal.

Legal aspects

In sending the manuscript the author(s) confirm(s) that (s)he has (they have) not previously submitted it to another journal (except for abstracts of no more than 400 words) or published it elsewhere. The author(s) also agree(s), if and when the manuscript is accepted for publication, to automatic and free transfer of copyright to the Publisher allowing for the publication and distribution of the material submitted in all available forms and fields of exploitation. The author(s) accept(s) that the manuscript will not be published elsewhere in any language without the written consent of the copyright holder, i.e. the Publisher.
All manuscripts submitted should be accompanied by an authors' statement including signed confirmation of the above and confirming that this publication has been approved by all co-authors (if any), as well as by the responsible authorities at the institution where the work has been carried out. The authors' statement should be signed by ALL co-authors. Additionally, the author(s) confirm(s) that (s)he is (they are) familiar with and will observe the "Instruction to Authors" included in *Folia Neuropathologica* and also that all sources of financial support have been fully disclosed. Materials previously published should be accompanied by written consent for reprinting from the relevant Publishers. In the case of photographs of identifiable persons, their written consent should also be provided. Any potential conflict of interest will be dealt with by the local court specific to the Publisher. Legal relations between the Publisher and the author(s) are in accordance with Polish law and with international conventions binding on Poland.
Authors agree to waive their royalties.

Anonymous review

All manuscripts will be subject to a process of anonymous editorial review.

Preparation of manuscripts

Articles must be written in English, with British spelling used consistently throughout. Authors not entirely familiar with English are advised to correct the style by professional language editors or native English speakers.
• The length of original article should not exceed 20 printed pages including text, illustrations, tables, and references.

- Manuscripts should be typed using 12pts.font, double-spaced, and fully corrected. Allow a margin at least 2.5 cm at the top, bottom and left side of the page. Text should not be justified.
- The title page should contain: the author's full names, title of the paper, all authors' affiliations, full name and address of the communicating author (including e-mail address and fax number), running title (not exceed 40 characters including spaces).
- The abstract should not exceed 350 words. A list of 3–10 key words is recommended below the abstract.
- The manuscript body should be organized in a standard form with separate sections: Introduction, Material and Methods, Results, Discussion, and References. Review articles should be divided into sections and subsections as appropriate without numbering.
- Do not underline in the text. Avoid footnotes.
- All dimensions and measurements must be specified in the metric system.
- The source of any drug and special reagent should be identified.
- Particular attention needs to be paid to the selection of appropriate analysis of data and the results of statistical test should be incorporated in the results section.
- The nomenclature used should conform to the current edition of the Nomina Anatomica or Nomina Anatomica Veterinaria.
- Acknowledgements should be made in a separate sheet following Discussion and before References. These should contain a list of dedications, acknowledgements, and funding sources.
- Legends of figures and tables should be typed on separate pages.
- The editor reserves the right to make corrections.

Tables

- Tables numbered in Roman numerals require a brief but descriptive heading.
- The major divisions of the table should be indicated by horizontal rules.
- Explanatory matter should be included in footnotes, indicated in the body of the table in order of their appearance.
- Tables must not duplicate material in the text or in illustration.

Illustrations

All figures should be supplied electronically at resolution 300dpi in all standard formats (tiff, jpg, Adobe Photoshop, Corel Draw, and EPS). Name your figure files with "Fig" and the figure number, e.g., Fig1.tif
- The maximum figure size is 84 mm or 174 mm for use in a single or double column width, respectively.
- When possible, group several illustrations on one block for reproduction. Like all other figures, block should be prepared within a rectangular frame to fit within a single or double column width of 84 and 174 mm, respectively, and a maximum page height of 226 mm.
- Each figure should include scale magnification bar; do not use magnification factors in the figure legends.
- All figures, whether photographs, graphs or diagrams, should be numbered using Arabic numerals and cited in the text in consecutive numerical order
- **Immunohistochemical study requires color illustrations of very good quality. The papers with white and black immunohistochemistry will not be accepted.**
- **For the paper accepted before 01.04.2016 the publication charge includes only the expense of color illustrations.** The cost of color print for every successive 8 pages is 200 euro irrespective of the number of color pages, i.e., the price remains the same whether there is one or eight pages.

The Publisher makes out the bill to the communicating Author.

References

The list of references (written on a separate page) should include only those publications that are cited in the text. Avoid citation of academic books, manuals and atlases. References may be arranged alphabetically and numbered consecutively. References should be given in square brackets with no space between the comma and the consecutive number, e.g. [3,4,6-12].

References should be written as follows:

Journal papers: initials and names of all authors, full title of paper, journal abbreviation (according to Index Medicus), year of publication, volume (in Arabic numerals), first and last page (example below):

1. Valverde F. The organization of area 18 in the monkey. Anat Embryol 1978; 154: 305-334.
2. Uray NJ, Gona AG. Calbindin immunoreactivity in the auricular lobe and interauricular granular band of the cerebellum in bullfrogs. Brain Behav Evol 1999; 53: 10-19.

Book and monographs: initials and names of all authors, full title, edition, publisher, place, year (examples below):

1. Pollack RS. Tumor surgery of the head and neck. Karger, Basel 1975.
2. Amaral DG, Price JL, Pitkanen A, Carmichael ST. Anatomical organization of the primate amygdaloid complex. In: Aggleton JP (ed.). The amygdala. Wiley-Liss, New York 1992; pp. 1-66.

Reference to articles that are accepted for publication may be cited as „in press" or Epub.

Proofs

Corrections to the proofs should be restricted to printer's errors only; other alterations will be charged to the authors. In order to maintain rapid publication, proofs should be returned within 48 hours, preferably by e-mail, fax or courier mail. If the Publisher receives no response from the authors after 10 days, it will be assumed that there are no errors to correct and the article will be published.

Subscription information

The journal is published in one volume per year consisting of four numbers. The annual subscription price is 160 PLN for Institutions from Poland and 80 PLN for individual subscribers from Poland and 140 Euro for foreign Institutions and 70 Euro for foreign individual subscribers.
Payment should be made to:
Termedia sp. z o.o., ul. Kleeberga 8, 61-615 Poznań
BZ WBK III O/Poznań PL 61 1090 1359 0000 0000 3505 2645
SWIFT: WBKPPLPP

The publisher must be notified of a cancellation of a subscription not later than two months before the end of the calendar year. After that date the subscription is automatically prolonged for another year.
Publishing, Subscription and Advertising Office:
TERMEDIA Publishing House
ul. Kleeberga 2
61-615 Poznań, Poland
phone/fax +48 61 822 77 81
e-mail: termedia@termedia.pl
http://www.folianeuro.termedia.pl

Folia Neuropathologica

AUTHOR'S STATEMENT

Title of the article

.....

.....

.....

The author(s) hereby confirm(s) that:

☐ The above-mentioned work has not previously been published and that it has not been submitted to the Publishers of any other journal (with the exception of abstracts not exceeding 400 words).

☐ All co-authors named and the relevant authorities of the scientific institutions at which the work has been carried out are familiar with the contents of this work and have agreed to its publication.

☐ In sending the manuscript together with illustrations and tables agree(s) to automatic and free transfer of copyright to the Publisher allowing for the publication and distribution of the material submitted in all available forms and fields of exploitation, without limits of territory or language, provided that the material is accepted for publication. At the same time the author(s) accept(s) that the submitted work will not be published elsewhere and in whatever language without the earlier written permission of the copyright holder, i.e. the Publisher.

☐ (S)he (they) agree to waive his(her)(their) royalties (fees).

☐ (S)he (they) empower(s) the Publisher to make any necessary editorial changes to the submitted manuscript.

☐ All sources of funding of the work have been fully disclosed.

☐ The manuscript has been prepared in accordance with the Publisher's requirements.

☐ (S)he (they) is (are) familiar with the regulations governing the acceptance of works as published in *Folia Neuropathologica* and agree(s) to follow them.

☐ (S)he (they) agree to accept appropriate invoice from the Publisher in case colour illustrations are implemented.

Date *Signatures of* ***all authors***

The covering letter formula can be found at: www.folianeuro.termedia.pl
-The covering letter should be sent to Associate Editor:

Milena Laure-Kamionowska
-Editorial Office of Folia Neuropathologica
Mossakowski Medical Research Centre, Polish Academy of Sciences
Poland Medical Research Centre
ul. Pawinskiego 5
02-106 Warszawa, Poland

CONTENTS