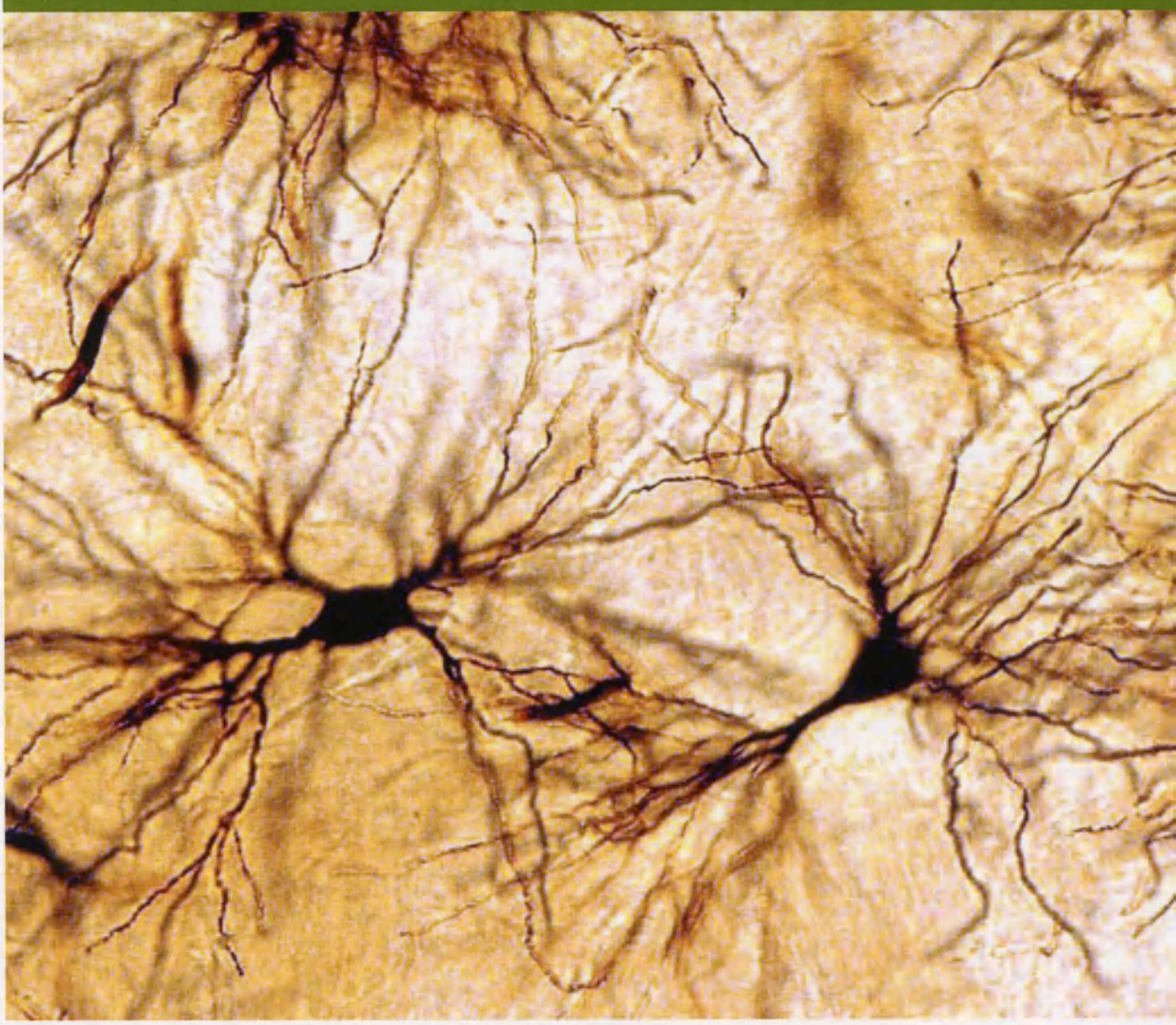
2016 / Volume 54 / Number 4
ISSN 1641-4640

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

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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; 54/4: 351-448

Contents

Review paper

Folia Neuropathologica

MicroRNAs as efficient biomarkers in high-grade gliomas

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Folia Neuropathol 2016; 54 (4): 351-359 DOI: 10.5114/fn.2016.64812

Abstract

High-grade gliomas are the most aggressive and devastating brain neoplasms. Therefore much effort is put on understanding their background as well as development of new effective diagnostic and therapeutic methods. However, until now the genetic only approach has not provided a satisfactory answer. Recently, it has been shown that the epigenetic issue is important for high-grade gliomas' development and progression. Out of many epigenetic mechanisms, as DNA methylation, histone methylation and acetylation, especially microRNAs showed to be deeply involved in the carcinogenesis process.

MicroRNAs are short non-coding RNAs. They are new candidates for human disease biomarkers due to their simple identification. MicroRNAs are stable in tissue and body fluids, what makes them very prospective non-invasive, blood-based biomarkers. There is a lot of data showing that various profiles of serum microRNAs are linked to numerous neoplastic processes, indicating that microRNAs can be really a new class of biomarkers for human diseases.

Key words: *microRNA, miRNA, miR, biomarker, high-grade glioma, HGG, glioblastoma, GBM.*

Introduction

Histopathology of tumour specimens gained by microsurgical resection or stereotactic biopsy is a standard diagnostic procedure for patients with high-grade gliomas (HGG). The neuropathological system classifies gliomas according to their morphological resemblance to the corresponding glial cells, cytoarchitecture and immunohistological properties [37]. However, the clinical course of the tumours comprising the same histopathological entity varies significantly. Magnetic resonance imaging is currently the best instrumental method for staging of the disease and follow up, but it reveals tumours only at a macroscopic level. It is clear that the effective management of any malignant neoplasm, and brain tumour particularly, requires a diagnosis at an earlier stage, and that states the need for specific and sensitive biomarkers. Although many potentially useful probes have already been suggested, no such markers have been established for brain tumours. In this review several specific microRNA biomarkers will be presented and discussed, including those identified by our group [47].

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Brain gliomas

Human brain cancers form a family of approximately 120 heterogeneous tumour entities and variants [37]. Their annual incidence accounts for 1.8% of all new cancer cases and the mortality reaches 2.3% of cancer deaths worldwide [26]. Gliomas account for over 70% of all primary brain tumours in adults, and they are divided into astrocytic, oligodendroglial, mixed, ependymal and embryonal types [37]. The astrocytic type occurs mainly in adults, including the most common (65%) and most malignant type – glioblastoma (GBM). That typically infiltrates into the adjacent cortex and through the corpus callosum into the contralateral hemisphere making itself surgically untreatable. From a histopathological point of view gliomas are classified by the cell of origin and grade. Grades I to IV are based on histology and clinical criteria [37]. Grade I is assigned to tumours with low proliferative potential, grade II covers diffusely infiltrative tumours with cytological atypia, grade III shows anaplasia and mitotic activity and in grade IV tumours additionally microvascular proliferation and/or necrosis is seen.

Malignant gliomas (HGGs, grade III and IV) are the most common type of primary brain tumours. Each year in more than 22,000 people in the United States a malignant glioma is detected [63], and most of that group will die within the first two years from diagnosis despite aggressive chemo- and radiotherapy. However, many of these relatively uniformly treated patients advance more quickly than others to recurrence and death. It is very rare for GBM patients to survive longer than 3 years [9]. Better prognosis is associated with several clinical and histopathological features, including young age, good performance status, gross total resection, adjuvant treatments, giant-cell subtype and oligodendroglial differentiation [25,37,56]. Glioblastoma patients differ in the clinical presentation and response to treatment because of a strong inter-tumoural heterogeneity coming from different gene mutations affecting signalling networks. Several molecular GBM subtypes have been identified [5]. The proneural subtype localizes typically in the frontal cortex, usually has *IDH1*/*IDH2* and *TP53* mutations, and demonstrates better prognosis and sensitivity to Notch inhibition [46] than the mesenchymal subtype, which is more aggressive, with greater vascularity, displaying more *NF1* lesions, and depending on TGF-β and NF-κB activity. The classical subtype is aggressive and frequently has *EGFR* lesions [46]. A fourth subtype, neural glioblastoma, is less well characterized.

The neuro-oncological practice depends strictly on tumour classification. Therefore therapies are applied in a relatively uniform way to all patients with a given histopathological diagnosis. Furthermore, classification constrains the scientific approach to brain neoplasia studies, with biological understanding based on presumptions about specific tumour types [24]. However, it is known that histologically identical tumours may have a very different outcome and response to treatment. Therefore cancer heterogeneity, both on a genetic and epigenetic level, has implications for therapy and shows challenges for the rational design of effective treatment rules [43]. Molecular markers with both diagnostic and prognostic potential contribute valuable tools by redefining tumour subtypes within each grade.

Biomarkers

A biomarker is a chemical compound specifically relevant to the disease, which can be applied to monitor the current neoplasm's state. The marker, despite having high diagnostic and prognostic values, may also suggest targeted therapies. Selective biomarkers can identify susceptibility risks and would be critical for establishing a proper time point for effective treatment. It is obvious that patients with early detected tumours have better chances for recovery and survival than those with advanced neoplasms at the time of diagnosis.

Molecular markers are changing the traditional classification by redefining tumour subtypes within each grade of malignancy and providing diagnostic and prognostic information. They become more and more an integral part of neurooncological practice. Biomarker status is already critical for clinical decisions in some gliomas' subtypes. For example, *IDH1/2* mutations show favourable prognosis across all glioma grades [58,62]. Chromosomal co-deletion of 1p/19q is positively predictive for chemotherapeutic response in anaplastic oligodendrogliomas [57]. O6-methylguanine-DNA methyltransferase (*MGMT*) promoter hypermethylation is a favourable prognostic marker in astrocytic high-grade gliomas [48]. It is also predictive for chemotherapeutic response in anaplastic gliomas and GBM [22,27,56].

MicroRNAs

MicroRNAs (miRNAs, miRs) form a family of small non-coding RNA molecules of 18-25 nucleotides that function in post-transcriptional gene regulation [54,55]. To date there have been ca. 2000 different human miRNAs referenced in the miRBase (release 21; June 2014). They are involved in a number of biological processes including cell proliferation, differentiation, developmental timing control, apoptosis and stress responses, as well as pathological states such as cancer [15,23].

miRNAs regulate gene expression through both translational repression and degradation of target messenger RNAs (mRNAs). The biogenesis of miRNAs involves two processes. The first one occurs in the nucleus, where the primary transcript (pri-miRNA) is processed into a precursor (pre-miRNA) by a nuclear RNase III (DROSHA). The second event takes place in the cytoplasm. The pre-miRNA is exported by exportin V from the nucleus and is cleaved by Dicer into a short-lived dsRNA of about 20-25 nucleotides. Then the double-strand is unwound and one strand forms the mature miRNA, which is incorporated into an Argonaut (Ago)-protein containing complex called the RNA induced silencing complex (RISC). The mature miRNA within the RISC recognises complementary sites in the 3'-UTR of target genes, what results in translational inhibition or destabilisation of the target mRNAs and downregulation of the encoded protein expression [19].

It is estimated that up to 50% of all human protein-coding genes are regulated by miRNAs. Each miRNA regulates up to hundreds of different mRNAs, and each mRNA is regulated by tents of miRNAs [55]. Their expression is population-, race-, and gender-dependent, as well as related to tissue state (healthy or diseased), and disease subtype [55]. MiRNAs are considered to be fundamental to normal cellular function in eukaryotes, and the alteration of microRNA expression and activity has been implicated in a variety of pathological processes. The greatest challenge for molecular medicine is while miRNAs regulate many mRNAs, they impact many proteins.

MiRNAs can be considered as cancer biomarkers when variations in their expression identify the cancerous state. Until now almost all analysed tumours have shown distinct miRNA profiles compared to normal tissues [38]. These profiles can be further associated with prognostic factors and disease progression [6,66].

MicroRNAs as biomarkers of gliomas

It has been shown that miRNAs are integrally involved in brain gliomas' development and progression [1,12]. They are essential regulators of many key pathways implicated in tumour pathogenesis [10,18]. Because miRNAs have been shown to play crucial roles in glioblastoma progression, invasion, tumour growth, and therapy responses, it is very likely that some miRNAs could be useful biomarkers in brain tumour patients.

MiRNAs expression can be altered in brain tumour through a variety of mechanisms including chromosomal changes, epigenetic defects and mutations in the machinery of their biogenesis [21,36]. There are many data showing that miRNA signatures can refine glioblastoma classification, differentiate GBM subclasses, as well as provide regulatory links to disrupted signalling pathways such as those facilitating cell growth [29]. Some studies show lower miRNA expression in tumour samples, what can be a function of cellular differentiation status [38]. Microarray studies of glioma tissue have implicated a number of miRNAs involved in glioma formation and propagation [47]. However, a comprehensive set of these differentially expressed RNA species has not been produced. Our group, based on miRNA microarray data analysis and deep RNA sequencing of miRNAs in normal human brain and tumour tissue, has recently found several RNA signatures, which were complemented with meta-analysis [47].

First we selected miRNAs that were most frequently deregulated in glioblastoma tissues as well as in peritumoral areas and compared with normal human brain. There were 22 differentially expressed miRNAs when comparing normal brain and brain tumour adjacent tissue [47]. The analysis revealed 6 miRNAs with elevated expression and 16 miRNAs with low expression within the tumour borders. 21 miRNAs found in the borders of gliomas were also identified in GBM. miR-625 was present within the border tissue, but not in GBM. Five overexpressed miRNAs, including the most abundant miR-21, are involved in the increased invasion and migration, but 15 were downregulated (Table I). We identified miRNAs associated with the progression from glioma grade III to grade IV. They are generally upregu-

Table I. Human microRNAs as high-grade glioma drivers, differentially expressed compared to adjacent tissue [7,47]

No.	Overexpressed	No.	Underexpressed
1.	21	1.	7
2.	142 –5p	2.	124
3.	155	3.	128
4.	221/222	4.	129*
5.	542 –5p	5.	129 –3p
6.	630	6.	132
7.	1260	7.	136
		8.	137
		9.	139 –5p
		10.	153
		11.	323 –3p
		12.	330 –3p
		13.	410
		14.	598
		15.	769 –5p
		16.	625

Table III. Differentially expressed human microRNAs in glioblastoma versus normal brain [7,47]

No.	Overexpressed	No.	Underexpressed
1.	9 –5p	1.	7 –5p
2.	10a –5p	2.	124 –3p
3.	10b –5p	3.	128 –3p
4.	15b –5p	4.	129 –5p
5.	16 –5p	5.	132 –3p
6.	17 –5p	6.	136 –5p
7.	20a –5p	7.	137
8.	21 –5p	8.	138 –5p
9.	25 –3p	9.	139 –5p
10.	92a –3p	10.	153 –3p
11.	92b –3p	11.	154 –3p
12.	93 –5p	12.	203a
13.	106a –5p	13.	218 –5p
14.	106b –5p	14.	323a –3p
15.	130a –3p	15.	328 –3p
16.	130b –3p		
17.	155 –5p		
18.	182 –5p		
19.	196 –5p		
20.	210		
21.	221/222		

lated (Table II). The most interesting is the panel of 35 differentially expressed miRNAs in glioblastoma versus normal human brain (Table III). There are 20

Table II. Potential new microRNA markers for gliomas' progression from grade III to IV [7,47]

No.	Overexpressed	No.	Underexpressed
1.	20a –3p	1.	33a
2.	134	2.	197
3.	144*	3.	340*
4.	150*	4.	381
5.	202	5.	574 –3p
6.	221/222		
7.	301b		
8.	378		
9.	483 –5p		
10.	494		
11.	500*		
12.	502 –3p		
13.	513a –5p		
14.	575		
15.	939		
16.	940		
17.	1202		
18.	1207 –5p		
19.	1224 –5p		
20.	1225 –5p		
21.	1246		
22.	1260		
23.	1275		
24.	1290		
25.	1471		
26.	1915		

and 15 miRNAs up- and downregulated, respectively. One can see the highly expressed miRNA-9, as well as miRNA-21 and 155, what is shown in Table I. They can be used as novel biomarkers and potential therapeutic targets for GBM. MiRNA-7, 124, 128, 129, 132, 136, 137, 139, 153 and 323 overlap with those downregulated listed in Table I. Differentially expressed miRNAs in brain tumour and adjacent tissue reflect the cross-talk between cancer cells and their local environment, which is a key feature of establishing and maintaining a malignant state. Future validation studies of these miRNAs, in combination with other brain tumour biomarkers hold a promise for clinical practice.

Candidate microRNAs for high-grade gliomas biomarkers

Regarding the up-to-date literature findings and our laboratory results we can characterize the most

promising of miRNA to be effective biomarkers for high-grade gliomas, especially GBM.

microRNA-21 shows overexpression in GBM and other gliomas in a grade-specific manner [4,7, 31,34,41,59]. It affects the major cancer pathways: TGF-β, p53, and mitochondrial initiated apoptosis pathways. The miR-21 knock down in glioma cell lines leads to upregulation of tumour suppressor proteins including p53, Bax, DAXX, APAF1, p21, TAp63 and TGFBR2 [45]. Other studies showed that miR-21 regulates matrix metalloproteinase inhibitors (RECK and TIMP3) therefore implicates tissue invasion [13].

microRNA-10b seems to be deeply involved in glioblastoma progression because its expression levels clearly correlate with tumour grade [50]. It is overexpressed in GBM [50,65] and enhances GBM invasiveness [50].

microRNA-221/222 were upregulated in gliomas and cell lines, but in HGGs with increased proliferation rates miRNA-221 levels were distinctly higher [7]. The direct link was found between miRNA-221/222 and cell cycle progression [42]. The ability of miRNA-221/222 to negatively regulate the pro-apoptotic gene PUMA is responsible for its anti-apoptotic effect [69]. The overexpression of PUMA changes the phenotypes caused by the overexpression of microRNA-221/222 [42,69], what suggests that miR-221/222 enhances the proliferative potential of tumour cells. The other way of miRNA-221 action in changing apoptotic signalling and altering cell cycle leads through BIRC family of neural cell fate regulators. It was shown that miRNA-221 is selectively upregulated in glioma tissue samples and cell lines, whereas its target, encoding the survivin-1 homolog BIRC1, a neuronal inhibitor of apoptosis protein (NIAP), is downregulated [40].

microRNA-17~92 cluster is upregulated in glioblastoma cell lines and tumour samples and is composed of miRNA-17-3p, -17-5p, -18a, -19a, -19b, -20a, and -92a [41]. The cluster targets are tumorigenic regulators of DNA-repair and angiogenesis (CTGF, and POLD2), and anti-proliferative transcripts (TGFBRII, SMAD4, and CAMTA1) [11,41].

Compared to non-neoplastic brain tissue **microRNA-451** is overexpressed in glioma cells [14,44] and regulates the adaptive response in metabolic stress and low glucose availability [16].

The other upregulated microRNA in highly aggressive GBM cell lines is **microRNA-145** [30]. That fact promotes decreased proliferation and invasion of GBM cell lines [33]. Oct4 and Sox2 are proved to be targets of miR-145 mediating the loss of "stemness".

One of the most commonly downregulated microRNAs in glioblastoma [17] and glioma cell lines, when compared to age-matched controls, is **microRNA-128** [4,34]. The downregulation of miRNA-128 inversely correlates with the WHO tumour grade. While miRNA-128 is downregulated in grade II–IV tumours, the levels in HGGs are significantly lower [69]. When overexpressed, miRNA-128 decreases the proliferative potential of glioblastoma cell lines *in vitro* and in GBM xenografts. miR-128 is also proposed to act by deregulation of self-renewal in glioma stem cells [17]. MiR-128 downregulation promotes an undifferentiated glioma phenotype via increased expression of its targets: angiopoietin-related growth factor protein 5 (ARP5, ANGPTL6), a transcription suppressor promoting stem cell renewal and inhibiting tumour suppressor genes expression involved in senescence and differentiation, Bmi-1, and a transcription factor critical for the control of cell-cycle progression, E2F-3a [8]. Addition of exogenous miRNA-128 to GBM cell lines restored the correct expression of those factors, and decreased the proliferation. Our data suggest that downregulation of miRNA-128 may contribute to glioma and GBM, in part, by co-ordinately upregulating ARP5 (ANGPTL6), Bmi-1 and E2F-3a, resulting in the proliferation of undifferentiated GBM cells [8].

The other commonly downregulated microRNA in GBM cell lines and tissues is **microRNA-34a**, which shows a significant reduction in p53-mutant cells compared to wild-type p53 cells [35,39]. miR-34a interacts e.g. with MYC, CCND1, CDK6, SIRT1 and c-Met oncogenes within the transcriptome [20] and plays an important role in gliomas by inhibiting glioma tumorigenesis as a tumour suppressor with silent information regulator 1 (Sirt1) as a negative target of miR-34a in glioma cell lines. Sirt1 is an regulating apoptosis oncogene in response to oxidative stress and genomic insults [39].

EGFR and Akt activated pathways are the most common genetic alterations in glioblastoma and act together in gliomagenesis. **microRNA-7**, downregulated in GBM [32,65], has been shown to inhibit EGFR expression, what leads to reduction in Akt phosphorylation [28]. The other targets of miRNA-7 are p21 activating kinase (PAK1) and focal adhesion kinase (FAK) microRNA-7. Therefore overexpression of microRNA-7 reduces GBM invasion and migration [3].

These data show the potential of miR-7 in the area of gliomas' therapeutics.

Compared to non-neoplastic brain tissue **microRNA-124/137** are downregulated in anaplastic astrocytomas and glioblastomas [53]. In tumour derived and neural stem cells they lead to G1 arrest and reduction in expression levels of CDK6, which is a regulator of the cell cycle and known target of miRNA-124 and -137. Moreover, miRNA-137, but not miRNA-124, is activated by addition of DNA demethylating agents to glioma cell lines and that suggests methylation of CpG islands based on miRNA-137 promoter regulation [53].

miR-181a and **miR-181b** are downregulated in glioma samples and cell lines [7,52] showing 20-30% reduction in glioblastomas. MiRNA-181b is a potential prognostic marker and helps in selection of patients who may benefit from adjuvant therapy.

microRNA-100 reduces proliferation and increases apoptosis of GBM lines by inhibiting the silencing mediator of retinoid or thyroid hormone receptor-2 (SMRT/NCOR2). Compared to normal neural cell controls it is downregulated in multiple GBM cell lines. MicroRNA-100 decreases proliferation in orthotopic GBM xenografts and extends survival [2].

Peripheral blood glioblastoma biomarkers

Monitoring of glioma development during or after completed treatment requires a reliable and quick test for biomarker detection from an easily accessible source, allowing a less extensive and more accurate disease monitoring in shorter time periods (as compared to neuroimaging) [64]. Furthermore, there could be a huge benefit from developing biomarkers for glioblastoma confirmation in order to avoid biopsy for patients with a high risk of surgery-associated mortality or small tumours in eloquent brain areas. The specific miRNA signature in plasma samples derived from GBM patients before tumour resection would be very useful for planning the necessary degree of resection and adjuvant therapies [64]. Circulating microRNAs and exosomal microRNAs may serve as non-invasive biomarkers for various diseases, also in different cancer types [60,64]. Circulating miRNAs are appealing biomolecules to be considered as cancer biomarkers for several reasons including their stability, which sustain a high temperature or extreme pH conditions that would damage other cell components.

A significant difference in serum miRNA profile was found between untreated high-grade astrocytomas (grade III-IV) and controls in a genome-wide miRNA analysis. Seven miRNAs (miR-15b*, miR-23a, miR-133a,miR-150*,miR-197,miR-497,miR-548b-5p) were markedly decreased in grade II-IV patients, and showed high specificity (97.87%) and sensitivity (88.00%) for prediction of malignant astrocytomas. Furthermore, these miRNAs were also elevated in serum after operation, and some of them could be proposed as non-invasive biomarkers of malignant and benign cases, astrogliosis and other primary brain tumours [67,68].

In comparison to normal controls the plasma levels of miR-21, miR-128 and miR-342-3p were shown to be significantly altered in GBM patients and miR-128 and miR-342-3p positively correlated with glioma' histopathological grade [61]. In blood of glioblastoma patients, compared with controls, miR-128 overexpression has been identified [49]. Unlike, in GBM tissue compared with normal human brain miR-128 was downregulated [17,70]. In the study comparing the blood samples obtained immediately after surgery versus more than 6 months after operation and after radio- and chemotherapy, miR-128 and miR-342-3p were deregulated likewise, what suggests that the detected differences are connected with the disease, not with a particular treatment [49]. Recently it has been shown that miR-454-3p in plasma of glioma patients is markedly overexpressed compared to healthy controls and are lower in LGGs than in HGGs. Also miR-29 shows high diagnostic potential, allowing to differentiate between patients of stage I-II with stage III-IV [68]. Furthermore, the miR-454-3p expression in the post-operative plasmas is markedly downregulated in comparison to the pre-operative plasmas, and a correlation of worsening prognosis of glioma was observed with increasing miR-454-3p expression [51]. Also a huge increase in miR-210 expression was found in GBM patients' serum samples compared to controls, and it was associated with the tumour grade and patient's poor outcome [68].

Conclusions

Altered miRNA biogenesis and expression in glioma plays a vital role in important signalling pathways associated with a range of tumour characteristics including gliomagenesis, invasion, and malignancy.

The progress in our understanding of the potential involvement of miRNAs in malignant gliomas is improving rapidly, hurdles remain high before miRNAs are recognized as valid markers in HGGs. The isolation and characterization of miRNA using cellular and molecular biology techniques from the circulation of glioma patients could potentially be used for improved diagnosis, prognosis, and treatment decisions.

Disclosure

Author reports no conflict of interest.

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Original paper

Folia Neuropathologica

Alzheimer's amyloid-β peptide disturbs P2X7 receptor-mediated circadian oscillations of intracellular calcium

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Folia Neuropathol 2016; 54 (4): 360-368 DOI: 10.5114/fn.2016.64813

Abstract

Recent data indicate that Alzheimer's disease (AD) is associated with disturbances of the circadian rhythm in patients. We examined the effect of amyloid-β (Aβ) peptide, the main component of the senile plaques playing a critical role in the deregulation of calcium (Ca^{2+}) homeostasis in AD, on the circadian oscillation of cytosolic calcium (Ca^{2+}) levels in vitro. The experiments we carried out in human primary skin fibroblasts. This cell line was previously shown to exhibit circadian rhythms of clock genes. Moreover, the basic clock properties of these peripheral cells closely mimic those measured physiologically and behaviorally in human and do not change during aging. In this study we showed that i) cytosolic Ca^{2+} oscillations depend on the activation of purinergic P2X7 receptors; and ii) these oscillations are abolished in the presence of Aβ. In total, our new findings may help to deepen our understanding of the molecular mechanisms involved in AD-related circadian alterations.

Key words: *amyloid-β, Alzheimer's disease, calcium, P2X7 receptors, circadian rhythms.*

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder that is characterized by progressive neuronal loss, mainly in the brain cortex and hippocampus [28]. One of the main neuropathological hallmarks of this disease is the deposition of extracellular senile plaques containing amyloid-β (Aβ) peptides, derived from processing of the amyloid-β precursor protein (APP) [3,18]. Many previous data point out to a critical role for deregulation of calcium (Ca^{2+}) homeostasis in the pathogenesis of AD. Thus, the levels of intracellular Ca^{2+} concentration and calcium-regulated enzymes (e.g. calpains, proteases, phospholipases) were found to be elevated in animal models of AD [24] as well as the brains of AD patients [47,48]. The "calcium hypothesis" was further supported by demonstrations that, during brain aging, the molecular processes responsible for Ca^{2+} regulation were impaired. This involved the mechanisms of Ca^{2+} sequestration into its intracellular stores (endo(sarco)plasmic-reticulum (ER) and mitochondria) as well as Ca^{2+} influx into the cytoplasm by voltage-gated Ca^{2+} channels, ionotropic or metabotropic receptors [13,21,64]. In AD, disturbed Ca^{2+} homeostasis is not restricted

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to neurons but represents a global phenomenon affecting virtually all cells in the brain. AD-related aberrant Ca^{2+} signaling in astrocytes and microglia probably contributes profoundly to an inflammatory response that, in turn, impacts neuronal Ca^{2+} homeostasis and brain function [8]. It has been shown that Aβ release induces intracellular calcium overload and activates intracellular calcium-dependent events, leading to a decrease in learning and memory as well as cognitive dysfunction [27,43,62]. Previous findings suggested that *N*-methyl-D-aspartate receptors (NMDARs) are the main mediators of enhanced Ca^{2+} entry evoked by Aβ [19,35]. However, more recent discoveries showed that the soluble form of Aβ oligomers induce their toxic effects by disrupting the integrity of the cell plasma membrane leading to uncontrolled fluxes of Ca^{2+} into the cells [66].

Moreover, recent data indicate that AD progression is associated with disturbances of the circadian rhythm in patients. Circadian rhythms govern a wide variety of physical, behavioral and metabolic processes that follow a roughly 24-hour cycle, responding primarily to the light/dark cycle. These are controlled by the circadian clock machinery, in which rhythm-generating mechanisms are encoded by a transcription-translation feedback loop. The mammalian circadian clock machinery is regulated by a central pacemaker in the suprachiasmatic nucleus (SCN) of the brain that synchronizes oscillators in peripheral tissue [16]. The entrained signals from SCN neurons are distributed through different target organs by efferent neural and humoral mechanisms, such as circulating melatonin, producing changes in metabolism, core body temperature, and sleep. Calcium ions are a potent second messenger coupling the clock gene oscillation and the rhythmic firing of action potential in SCN neurons [33,37,58,60]. Calcium mediates intracellular clock signals, such as entrainment processes [6,23,30], clock gene expression [33,37,45,55], and output signaling [2]. Moreover a topological specificity of the circadian Ca^{2+} rhythm in SCN was observed, suggesting that calcium plays a role in the hierarchical organization of rhythmicity in the central pacemaker [25].

The alterations of the SCN as well as in melatonin secretion are the major factors of circadian clock disruption [72]. Insomnia, nocturnal behavioral changes, sundowning syndrome and excessive daytime sleepiness are the common to circadian disturbances observed in AD patients as well as patients with mild cognitive impairment [7,17,67,68,70]. The studies in animal and humans demonstrated that Aβ level in the cerebrospinal fluid is modulated by sleep-wake cycles [5,36,40]. This raises the possibility that disturbances in the circadian rhythm causes brain Aβ accumulation over time, suggesting a causative rather than an associative link between sleep loss and Aβ accumulation [52]. However early-stage AD events such Aβ aggregation and disturbances in calcium homeostasis may also induce molecular changes that lead to circadian clock disruption. Therefore the aim of this study was to deepen our understanding of the molecular mechanisms involved in AD-related circadian clock alterations, by investigating i) the clock-dependent regulation of intracellular calcium levels in a peripheral tissue, and; ii) the effect of Aβ peptides on the changes of cytosolic calcium levels around the clock. For this purpose, we used primary cultures of human fibroblasts because: i) fibroblasts coming from AD patients present a disturbed Ca^{2+} homeostasis [31,39]; and ii) fibroblasts are a valuable *in vitro* model of peripheral oscillators [10,56,57].

Material and methods

Ethical permission

Prior ethical consent to the use of human skin tissues was given by the Ethical Committee of Basel, and informed written consent to participation in this study was obtained from all human subjects.

Materials

Human recombinant $A\beta_{42}$ was purchased from Bachem (Germany). Coomassie Brilliant Blue G, DMSO, DMEM, Fetal Bovine Serum (FBS), Glutamax, Hank's Balanced Salt Solution (HBSS), Horse Serum, Penicillin/Streptomycin, Pluronic® F-68, Thapsigargin and ATP were obtained from Sigma (St. Louis, MO, USA). Liberase was from Roche Diagnostics GmbH (Mannheim, Germany). Fluo-4 AM was obtained from Invitrogen (Thermofisher Scientific, Waltham, Massachusetts, USA).

Tissue isolation and fibroblast culture

Two cylindrical cutaneous biopsies (2 mm diameter) were taken from the buttocks of a healthy male subject. Fibroblasts were isolated from biopsies by

4-h digestion of tissue in DMEM/1% penicillin streptomycin/1% Glutamax (DMEMc)/20% FBS/87.5 ng/ml Liberase, and cultured in DMEMc/20% FBS.

Synchronization of cells and timetable to study cellular circadian rhythms

For all experiments, cells were seeded onto collagen-coated 48-well or 96-well dark plates at the density of $1,4*10^5$ cells per ml. Cells were synchronized by treatment with DMEM containing 50% Horse Serum for 2 hours at 37°C. After the synchronization, cells were washed with PBS and the medium was changed into DMEM/2% FBS according to [1]. Experiments were performed every 4 hours, starting 4 h after synchronization and until 48 h.

Preparation of Aβ species and cell treatment

$A\beta_{42}$ was dissolved in PBS to make stocks of 500 µM and stored at –80°C until use. Aging of the peptides was induced by shaking the diluted solution (50 µM) at 1000 rpm overnight at 37°C. The cells were treated after synchronization at a final concentration of 0.5 µM of $A\beta_{42}$. In selected experiments, after measurement of basal Ca^{2+} levels, cells were treated either with a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor (Thapsigargin; 10 nM), or with an agonist (ATP; 1 mM) of purinergic P2X7 receptors. The measurements were repeated immediately or after 5 min of incubation, respectively.

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ measurement was carried out using the fluorescent indicator Fluo-4 acetoxymethyl (AM) ester (200 µM stock solution in DMSO). At the specific time points after cell synchronization, fibroblasts were loaded with 4 µM Fluo-4 AM supplemented with 0.02% Pluronic® F-68 for 60 min at 37°C in a standard HBSS. The cells were washed 3 times with HBSS and, to ensure complete AM ester hydrolysis, kept for 30 min at 37°C in the dark. After a second washing step, the fluorescence was measured using Fluoroskan® counter at 485/520 nm. To study the involvement of purinergic receptors and endoplasmic reticulum (ER) stores on a cytosolic calcium level, cells were treated with an agonist (ATP, 1 mM) or an antagonist of purinergic P2X7 receptors (Coomassie Brilliant Blue G, 5 µM) for 1 min and a non-competitive SERCA inhibitor for 30 seconds, as described in figures' captions, and the fluorescence of Fluo-4 was measured.

Statistical analysis

The results were expressed as mean values ± SEM. Differences between means were analyzed using Student's two-tailed *t* test. $P < 0.05$ was considered statistically significant. Cosinor software analysis was used to evaluate and estimate the parameters of circadian rhythm (period plus mean, amplitude and acrophase).

Results

Since intracellular calcium was previously shown to exhibit a well-defined circadian rhythm in neuronal population of SCN [32], we first verified whether Ca^{2+} level exhibited a circadian rhythmicity in peripheral oscillators (Fig. 1). We used human primary fibroblasts since these cells are an excellent *in vitro* model of peripheral oscillators [10,56,57] and show a disturbed Ca^{2+} homeostasis in AD patients [31,39].

In the current study, fibroblasts presented changes in Ca^{2+} accumulation, with a peak 16 h post-synchronization (TP16) and a trough 28 h post-synchronization (TP28) (Fig. 1A). Under these conditions, relative Fluo-4 fluorescence in cell cultures was significantly different ($p < 0.001$) between the peak and the trough (Fig. 1D).

Since endoplasmic reticulum (ER) Ca^{2+} stores are important in the regulation of Ca^{2+} signaling in cells, we quantified $[Ca^{2+}]_i$ in fibroblasts after treatment with 10 nM thapsigargin (THAPS), an SERCA inhibitor. Depletion of ER Ca^{2+} stores with THAPS did not alter the circadian rhythm of $[Ca^{2+}]_i$ but increased the total Ca^{2+} level (Fig. 1B). The significant difference between $[Ca^{2+}]_i$ at TP16 and TP28 was still present (Fig. 1D).

Considering the controlling mechanisms of cytosolic Ca^{2+} fluctuations, it is possible that receptor-mediated Ca^{2+} influx is involved in the regulation of circadian rhythm of $[Ca^{2+}]_i$. Since primary human fibroblasts are electrically non-excitable and do not express voltage-gated Ca^{2+} channels, Ca^{2+} could be transported via purinergic P2X receptors, especially P2X7 subtype that is widely distributed in skin tissue [65]. Using the specific antagonist of P2X7 receptor-Brilliant Blue G (BBG), we showed that treatment with this compound abolished circadian oscillations

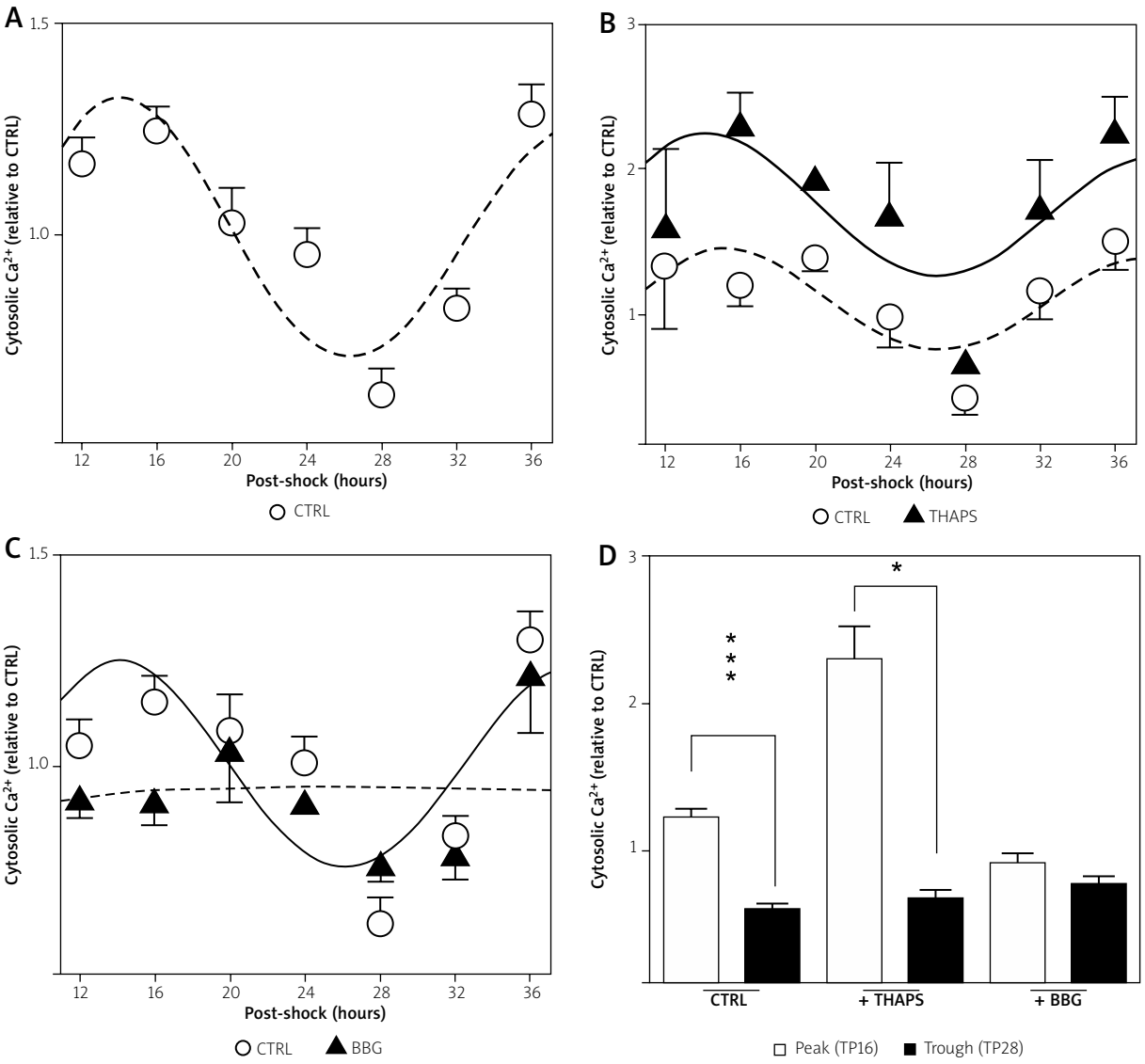

Fig. 1. Circadian oscillations of cytosolic calcium depend on activation of P2X7 receptors but not calcium uptake through SERCA. **A)** Cytosolic calcium levels were evaluated by using fluorescent dye, Fluo-4 (4 µM), in synchronized human skin fibroblasts from 12 hours post-synchronization time point every 4 hours for 7 time points (n = 5). **B-C)** Cytosolic calcium levels were evaluated by using fluorescent dye, Fluo-4 (4 µM), in synchronized human skin fibroblasts from 12 hours post-synchronization time point every 4 hours for 7 time points in presence of **(B)** thapsigargin (THAPS, 10 nM), inhibitor of SERCA, or **(C)** Coomassie Brilliant Blue G, antagonist of purinergic P2X7 receptors (BBG, 5 µM). **D)** Relative cytosolic calcium level at 16 hours post-shock (peak: TP16) and at 28 hours (trough: TP28) compared to non-treated cells (CTRL) (n = 3-5). The emitted fluorescence is linearly related to the cytosolic calcium content. *p < 0.05, ***p < 0.001; Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

of $[Ca^{2+}]_i$ (Fig. 1C). Thus, the significant difference between $[Ca^{2+}]_i$ at TP16 and TP28 was not observed anymore (Fig. 1D).

Disturbances of the Ca^{2+} homeostasis have been demonstrated to be associated with Aβ neurotoxicity. Therefore, we investigated the effect of Aβ peptides on the circadian fluctuation of Ca^{2+} levels (Fig. 2). Our data showed that extracellular treatment with $A\beta_{42}$ (aged peptide) completely abolished circadian oscillations of intracellular Ca^{2+} and impacted the levels of $[Ca^{2+}]_i$ at

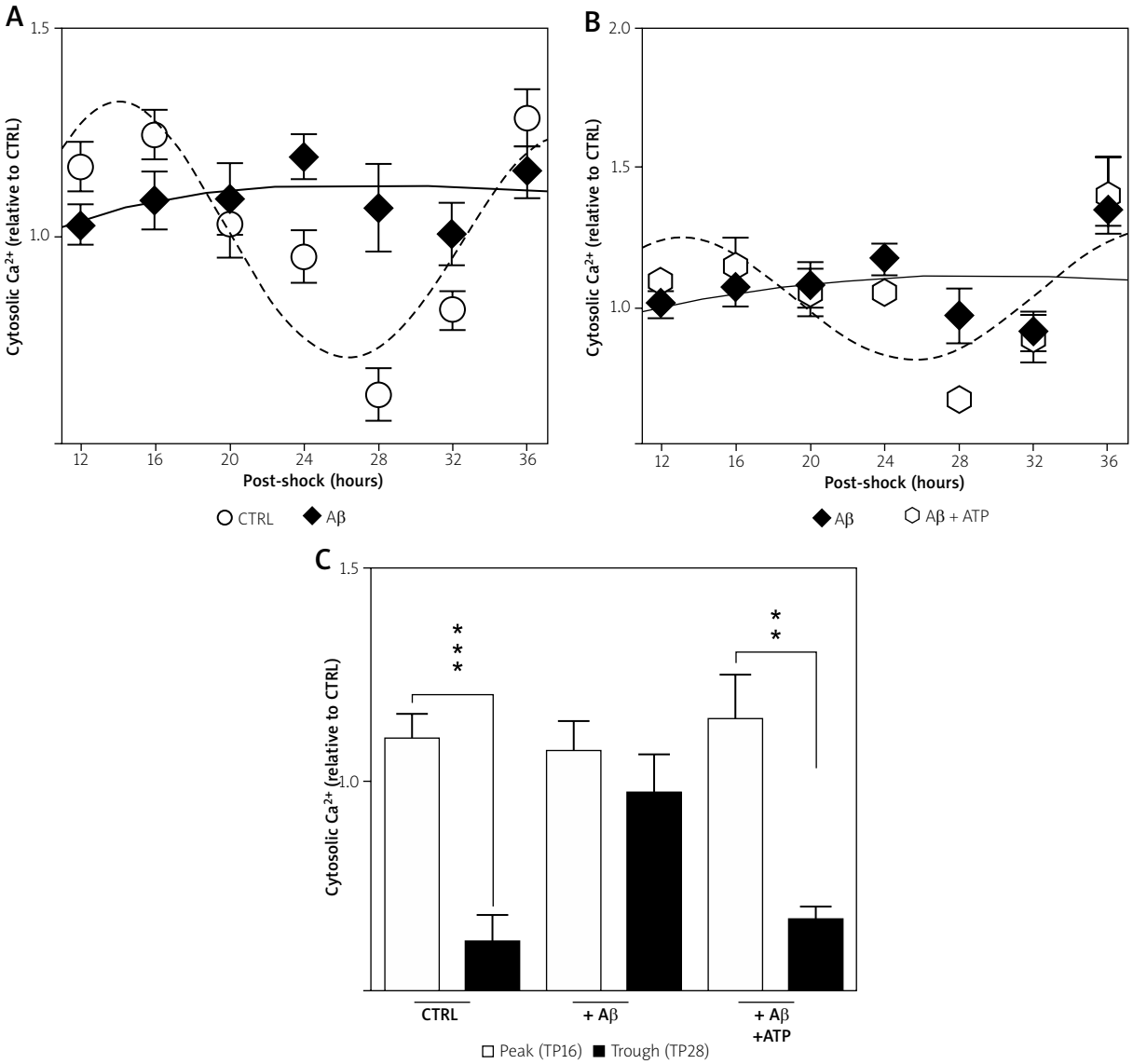

Fig. 2. Cytosolic calcium oscillations are abolished in the presence of Aβ. **A-B)** Cytosolic calcium levels were evaluated by using fluorescent dye, Fluo-4 (4 μM), in synchronized human skin fibroblasts from 12 hours post-synchronization time point every 4 hours for 7 time points in presence of **(A-B)** $A\beta_{42}$ (aged peptide) at 0.5 μM (n = 3). **(B)** Aβ treated cells were co-treated with ATP (1 mM) to overcome the dampening due to Aβ presence (n = 3). **(C)** Relative Cytosolic Calcium level at 16 hours post-shock (peak: TP16) and at 28 hours (trough: TP28) compared to non-treated cells (CTRL) (n = 3). Aβ treatment completely abolished differences in $[Ca2+]_i$ concentration between peak and trough time points while ATP treatment rescued the circadian oscillation of cytosolic calcium. The emitted fluorescence is linearly related to the cytosolic calcium content. $^{**}p < 0.01$, $^{***}p < 0.001$; Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

peak and trough time points (Fig. 2A and 2C). Those Aβ-evoked disturbances were not mediated by deregulation of ER Ca^{2+} stores, because THAPS treatment did not restore rhythmic oscillations of $[Ca^{2+}]_i$ (Supplementary Figure 3 online). Interestingly, treatment with adenosine triphosphate (ATP) abolished the negative effect of Aβ and restored oscillation of $[Ca^{2+}]_i$ (Fig. 2B and 2C). These data suggest that Aβ peptides altered the P2X7 receptor-mediated circadian rhythm of $[Ca^{2+}]_i$, leading to disturbances of calcium homeostasis.

Discussion

Accumulating evidence has suggested that sleep disturbances may be early indicators of dementia and may actually precede the onset of cognitive symptoms in AD [53]. Moreover, the sleep–wake cycle was shown to be a critical regulator of Aβ release and loss of slow wave sleep resulted in higher cumulative levels of neuronal activity and higher Aβ concentration in CSF [12]. It was previously suggested that intracellular Ca^{2+} may be a coordinator of the circadian timing system and biochemical reactions due to its ubiquitous role as a metabolic regulator [4]. Therefore the disturbances in Ca^{2+} homeostasis observed in AD brains could be partly associated with deregulation of patients' circadian rhythms. However the role of Ca^{2+} in regulating the clock function in pathophysiology is unknown. In this study, we showed for the first time that Alzheimer's Aβ peptides could negatively influence circadian fluctuations of Ca^{2+} in peripheral oscillators. This subsequently may alter calcium-dependent molecular processes involved in circadian clock regulation in AD.

It was demonstrated that intracellular Ca^{2+} concentration exhibits circadian rhythms in pacemaker neurons of the SCN [15,38]. The oscillatory physiology of Ca^{2+} was shown to be regulated by the circadian fluctuations in the Ca^{2+} currents generated by voltage-dependent calcium channels (VDCC) [41,60]. Calcium fluctuations were also shown in astrocytes of SCN, but, unlike in neurons, they were regulated by the Ca^{2+} release from ER stores [11,20]. Our study extends previous results by showing the existence of daily fluctuations in cytosolic calcium in peripheral oscillators, the human skin fibroblasts. This cell line was previously shown to exhibit circadian rhythms of clock genes, and the clock properties of these peripheral cells closely mimic those measured physiologically and behaviorally in humans [10,57]. Therefore, skin fibroblasts are a good *in vitro* model for studying molecular mechanisms of circadian rhythms. Moreover, it was shown that aging does not alter the basic clock properties (period length, amplitude and phase) of fibroblasts [56]. We observed that calcium oscillations in fibroblasts correspond to the previously demonstrated changes in transcript levels of the clock genes Per2, Bmal1, Rev-Erb, and Cry1 in those cells [9]. Former data showed that Ca^{2+} mobilized from internal deposits modulates the molecular circadian clock of hepatic cells *ex vivo*, in a manner that did not depend on the entrainment cue (meal or light) [4]. This suggests that Ca^{2+} signaling is a key regulator of circadian rhythms in peripheral tissues in contrast to the central pacemaker mediating hierarchical organization of rhythmicity [25].

Calcium signaling in non-excitable cells is initiated by mobilization of Ca^{2+} from intracellular ER stores through IP3 and ryanodine receptors. In our experiments, inhibition of SERCA significantly elevated cytosolic Ca^{2+} level, but did not alter calcium fluctuations. A similar effect was observed in SCN astrocytes [11]. Besides, in SCN neurons, the expression of SERCA was shown to follow a circadian pattern [50]. Together, all these data suggested that ER stores are not necessary for controlling $[Ca^{2+}]_i$ daily oscillations in non-neuronal cells.

Previous data reported the crosstalk between circadian oscillation of intracellular Ca^{2+} and rhythmic extracellular ATP accumulation in SCN astrocytes [11]. Exogenous ATP was shown to be a mediator of intercellular communication in physiology and neurodegeneration, by acting on the cell surface receptors, including ligand-gated ion channels (P2X) and G-protein coupled (P2Y) receptor subtypes. It was demonstrated that ATP selectively promotes the expression of the clock gene Per1 through gene transactivation after stimulation of P2X7 purinergic receptors in microglial cells [54]. Moreover, the endogenous purinergic receptors were shown to determine the local clock activity in the urinary bladder cells [69]. Therefore, the ATP-signaling may be also involved in changes of Ca^{2+} fluctuations in peripheral oscillators. Indeed, our study demonstrated that the circadian rhythm of the calcium level in fibroblasts depend on activation of the ATP-binding receptor, P2X7.

In AD pathology, changes of neuronal Ca^{2+} concentration are responsible for the oxidative stress as well as altered metabolism of APP and overproduction of Aβ peptides. On the other hand, Aβ neurotoxicity has been associated with the disturbances of intracellular Ca^{2+} homeostasis in neurons as well as in glial cells. The studies using APP transgenic mouse models of AD identified significantly elevated numbers of neurites with overloaded cytosolic Ca^{2+} and this effect was positively correlated with the distance from Aβ plaques [43]. Many previous studies demonstrated impaired Ca^{2+} regulation in fibroblasts of AD patients [31,39]. Our study confirms those reports, by demonstrating that exposure

to $A\beta_{42}$ (aged peptide) abolished Ca^{2+} fluctuations in the cytosol of fibroblasts.

A disruption of the Ca^{2+} regulation in the ER was previously shown to mediate signal-transduction alterations associated with AD [44]. Moreover, mutations that cause familial Alzheimer's disease have been linked to disturbances in intracellular calcium signaling pathways [34]. Skin fibroblasts from humans that harbor a mutation in presenilin 1 (PS1-A246E) showed exaggerated Ca^{2+} release from IP3-gated stores compared to controls after treatment with bombesin and bradykinin [39]. The elevated Ca^{2+} release from the ER, evoked by activation of the IP3 [14] or ryanodine [61] receptors, was shown to increase Aβ level. Overexpression of SERCA was also shown to increase Aβ production [29]. Furthermore, ER is also a potential intracellular target for Aβ protein [26,71], which disrupts the function of the intracellular Ca^{2+} stores. In our study, thapsigargin treatment did not restore the physiological oscillations of $[Ca^{2+}]_i$ that were significantly altered by Aβ. These data suggest that Aβ-mediated disruption of intracellular Ca^{2+} homeostasis may be evoked by an excess of calcium influx across the plasma membrane.

Furthermore, previous studies indicated that an altered activity of the purinergic P2X7 receptor mediates the pro-inflammatory processes in a transgenic AD model and in brains of AD patients [46,49,59]. The observation that Aβ may cause ATP release from microglia, and that P2X7 receptor is an obligate participant in microglia activation by Aβ, put the role of ATP and P2 receptors as a key event in neurodegeneration [42,63]. Recent data demonstrated that *in vivo* inhibition of P2X7 receptors significantly reduces the amyloid plaques formation in brain hippocampal structures through activation of α-secretase activity [51]. The mechanism of P2X7R-specific cleavage of APP was shown to be independent of ADAM9, -10, and -17 activity, but involved Erk1/2 and JNK phosphorylation [22]. In our study, we demonstrated that Aβ peptide significantly interferes with the P2X7-receptor mediated circadian oscillations of intracellular Ca^{2+}, however the mechanism underlying this phenomenon needs to be further investigated.

In summary, our data provide first evidence that Alzheimer's $A\beta_{42}$ peptides induce disturbances of P2X7 receptors mediated Ca^{2+} oscillation in peripheral oscillators. These findings may be therefore helpful for a better understanding of the circadian rhythms disruption related to AD.

Acknowledgments

Financial support was provided by the following grants: Sciex-NMSch to A.W. as well as Swiss National foundation (SNF #310030_122572) and Synapsis Foundation to A.E. The sponsors of this study were not involved in the study design; in collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Disclosure

Authors report no conflict of interest.

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Original paper

Folia Neuropathologica

Combined use of biochemical and volumetric biomarkers to assess the risk of conversion of mild cognitive impairment to Alzheimer's disease

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Folia Neuropathol 2016; 54 (4): 369-374 DOI: 10.5114/fn.2016.64815

Abstract

Introduction: *The aim of our study was to evaluate the usefulness of several biomarkers in predicting the conversion of mild cognitive impairment (MCI) to Alzheimer's disease (AD): β-amyloid and tau proteins in cerebrospinal fluid and the volumetric evaluation of brain structures including the hippocampus in magnetic resonance imaging (MRI).*
Material and methods: *MRI of the brain with the volumetric assessment of hippocampus, entorhinal cortex, posterior cingulate gyrus, parahippocampal gyrus, superior, medial and inferior temporal gyri was performed in 40 patients diagnosed with mild cognitive impairment. Each patient had a lumbar puncture to evaluate β-amyloid and tau protein (total and phosphorylated) levels in the cerebrospinal fluid. The observation period was 2 years.*
Results: *Amongst 40 patients with MCI, 9 (22.5%) converted to AD within 2 years of observation. Discriminant analysis was conducted and sensitivity for MCI conversion to AD on the basis of volumetric measurements was 88.9% and specificity 90.3%; on the basis of β-amyloid and total tau, sensitivity was 77.8% and specificity 83.9%. The combined use of the results of volumetric measurements with the results of proteins in the cerebrospinal fluid did not increase the sensitivity (88.9%) but increased specificity to 96.8% and the percentage of correct classification to 95%.*

Key words: *mild cognitive impairment, conversion, biomarkers, volumetry, β-amyloid, Alzheimer's disease, cerebrospinal fluid, magnetic resonance imaging, hippocampus.*

Introduction

Mild cognitive impairment (MCI) was treated in the past as a transitional state between the physiological aging and dementia. Currently it is a separate diagnosis, although very heterogeneous. It requires clinical vigilance because of possibility of conversion to dementia, most often to Alzheimer's disease (AD), with an average of 7-15% per year. The moment of conversion is very important due to the possibility of therapeutic effects, which are most effective in the early stages of AD, while the recommended treatment of MCI does not exist. Criteria for diagnosis of AD (NIA/AA, 2011) [1] include not only the dementia phase but also the MCI phase and preclinical phase

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of Alzheimer's disease pathophysiological process, when pathological changes are present in the brain but the patient does not have any clinical symptoms. Such state can last for even twenty years.

Although "amyloid cascade hypothesis" has given rise to doubts [2], diagnostic criteria of MCI from 2011 indicate the important role of biomarkers [1]. Biomarkers can improve the prediction of MCI conversion to AD. Significant markers include markers of β-amyloid (Aβ) deposition (decreased level of $A\beta_{1-42}$ in the cerebrospinal fluid (CSF) or positive amyloid imaging in PET) and markers of neuronal injury (increased levels of tau protein- total and/or phosphorylated in CSF or decreased glucose uptake in the temporal-parietal area in FDG-PET or reduced volume of hippocampus in magnetic resonance imaging – MRI) [1]. Currently, these parameters are not used in clinical practice because of the lack of treatment of MCI due to AD. However, positive biomarkers increase the likelihood that the cognitive impairment can be caused by the pathophysiological process of AD [9]. In such case the probability of MCI conversion to AD in the future is higher.

The aim of our study was to evaluate the usefulness of several biomarkers in predicting the conversion of MCI to AD: β-amyloid and tau proteins in the CSF and volumetric evaluation of different brain structures including the hippocampus in MRI.

Material and methods

The study population was 40 patients (22 women and 18 men), aged 50-80 years, with MCI diagnosed in the Alzheimer's Department (according to the diagnostic criteria from 2004; Winblad *et al.*) [16]. The Mini Mental State Examination (MMSE) [7], neurological and neuropsychological assessments (using standard neuropsychological tests) were performed; on CDR scale all patients received 0.5 [10]. Laboratory tests were taken to exclude other causes of cognitive impairment. Brain MRI was performed for all patients on a 1.5 T Toshiba apparatus to calculate volumes of selected structures (hippocampus, entorhinal cortex, posterior cingulate gyrus, parahippocampal gyrus, superior, medial, inferior temporal gyri and total intracranial volume) using FreeSurfer software. Each volume (hippocampus, entorhinal cortex, posterior cingulate gyrus, parahippocampal gyrus, superior, medial, inferior temporal gyri) was divided by the total intracranial volume to normalize results and to eliminate differences in the brain size (according to Whitwell) [15]. All volumes were multiplied by 1000 in order to facilitate comparison between them. Each patient had a lumbar puncture to evaluate Aβ and tau protein (total and phosphorylated) in the cerebrospinal fluid. There was a 2-year observation period. During control visits, MMSE, neurological and neuropsychological examinations were performed to assess potential disease progression to AD. Alzheimer's disease was recognized on the basis of the diagnostic criteria NIA/AA, 2011 [1].

Patients diagnosed with conversion to AD had been treated with the acetylcholinesterase inhibitor. All patients have remained under the care of our Memory Disorders Outpatient Clinic and have had periodical follow-up visits.

Table I. Characteristics of patients in studied subgroups with regard to Alzheimer's disease biomarkers concentration in cerebrospinal fluid

Variable	MCI whole sample	MCI stable	Converters
N	40	31	9
Age	63.17 (9.56)	61.26 (8.61)	69.78 (10.23)
MMSE	27.50 (1.73)	27.58 (1.79)	27.22 (1.56)
Years of education	13.95 (2.88)	14.13 (2.74)	13.33 (3.43)
$A\beta_{1-42}$	607.873 (269.92)	653.026 (242.96)	452.344 (314.16)
tTau	299.776 (196.64)	269.355 (166.12)	404.561 (262.82)
pTau 181	45.480 (19.94)	43.145 (19.03)	53.522 (22.08)
$A\beta_{1-42} \leq 609.54$	20	13 (41.9%)	7 (77.8%)
tTau ≥ 277.02	17	11 (35.5%)	6 (66.7%)
pTau 181 ≥ 55.08	10	7 (22.6%)	3 (33.3%)

Data presented as mean (standard deviation)
$A\beta_{1-42}$ – CSF amyloid beta 1-42 (pg/ml), tTau – CSF total tau (pg/ml), pTau 181 – CSF hyperphosphorylated tau at threonine 181 (pg/ml)

Table II. Descriptive statistics in each subgroup (normalized volumes were multiplied by 1000)

Structure	Non-converters (n = 31)		Converters (n = 9)		All (n = 40)	
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation
LH	2.529	0.253	2.009	0.418	2.412	0.365
RH	2.528	0.347	2.138	0.391	2.440	0.389
LERC	0.596	0.138	0.447	0.128	0.562	0.149
RERC	0.479	0.125	0.411	0.084	0.464	0.120
LPCG	1.613	0.253	1.549	0.287	1.599	0.259
RPCG	1.649	0.317	1.55	0.274	1.627	0.308
LPHG	1.109	0.158	1.04	0.252	1.094	0.182
RPHG	1.051	0.164	1.007	0.224	1.041	0.177
LITG	5.794	0.777	5.167	1.144	5.653	0.896
LMTG	5.432	0.591	5.216	0.991	5.383	0.692
LSTG	6.111	0.814	5.567	1.168	5.988	0.918
RITG	5.903	0.872	5.153	0.839	5.734	0.911
RMTG	6.128	0.88	5.747	1.182	6.042	0.982
RSTG	6.01	0.921	5.495	0.922	5.894	0.904

LH – left hippocampus, RH – right hippocampus, LERC – left entorhinal cortex, RERC – right entorhinal cortex, LPCG – left posterior cingulate gyrus, RPCG – right posterior cingulate gyrus, LPHG – left parahippocampal gyrus, RPHG – right parahippocampal gyrus, LITG – left inferior temporal gyrus, LMTG – left medial temporal gyrus, LSTG – left superior temporal gyrus, RITG – right inferior temporal gyrus, RMTG – right medial temporal gyrus, RSTG – right superior temporal gyrus

Table III. Results of a Student's *t*-test

	The value of *t* statistics	Degree of freedom (df)	Significance (two-sided)	Average difference
LH	–3.549	10	**0.005**	–0.52
RH	–2.891	38	**0.006**	–0.39
LERC	–3.022	14	**0.009**	–0.15
RERC	–1.541	38	0.132	–0.07
LPCG	–0.652	38	0.519	–0.06
RPCG	–0.855	38	0.398	–0.10
LPHG	–0.998	38	0.325	–0.07
RPHG	–0.655	38	0.516	–0.04
LITG	–1.910	38	0.064	–0.63
LMTG	–0.820	38	0.417	–0.22
LSTG	–1.596	38	0.119	–0.54
RITG	–2.289	38	**0.028**	–0.75
RMTG	–1.027	38	0.311	–0.38
RSTG	–1.532	38	0.134	–0.52
β-amyloid	–2.042	38	**0.048**	–200.7
Total tau	1.873	38	0.069	135.2
Phosphorylated tau	1.390	38	0.172	10.4

LH – left hippocampus, RH – right hippocampus, LERC – left entorhinal cortex, RERC – right entorhinal cortex, LPCG – left posterior cingulate gyrus, RPCG – right posterior cingulate gyrus, LPHG – left parahippocampal gyrus, RPHG – right parahippocampal gyrus, LITG – left inferior temporal gyrus, LMTG – left medial temporal gyrus, LSTG – left superior temporal gyrus, RITG – right inferior temporal gyrus, RMTG – right medial temporal gyrus, RSTG – right superior temporal gyrus

Results

Amongst 40 patients with MCI, 9 (22.5%) converted to AD within 2 years of observation (on average 9.2 months, SD 5.8). The study population was divided into two subgroups: subgroup 1: non-converters, who did not convert to AD (31 patients) and subgroup 2: converters, who converted to AD (9 patients). The characteristics of subgroups, including the results of CSF are shown in Table I, together with the cut-off points (established in our laboratory, described in our previous study [8]).

On the basis of our laboratory cut-offs the most corresponding was $A\beta_{1-42}$ value, which was positive for 7 of 9 (77.8%) converters but for 13 of 31 non-converters it was false positive. A positive value for total tau protein was obtained for 6 of 9 (66.7%) converters and the value false positive for 11 patients with stable MCI. The result of phosphorylated tau protein was positive only for 33% of converters. Table II presents descriptive statistics for all measured structures in MRI – average normalized values were multiplied by 1000 for easier data comparison.

Table III shows the results the Student's *t*-test significance of differences between subgroups for independent samples.

Statistically significant values were obtained for the left hippocampus, right hippocampus, left entorhinal cortex, right inferior temporal gyrus and Aβ ($p \leq 0.05$). Discriminant analysis model used all volumetric measurements and values of Aβ and total tau to determine subgroup membership: converter or non-converter. Discriminant analysis was conducted in three steps: for volumetric measurements only, for Aβ and total tau (phosphorylated tau was excluded because of high *p*-value) and for volumetry and CSF biomarkers. Sensitivity for MCI conversion to AD on the basis of volumetric measurements was 88.9% and specificity 90.3%. On the basis of Aβ and total tau sensitivity was 77.8% and specificity 83.9%. The percentage of correct classification using the results of the volumetric measurement was 90%, and by using Aβ and total tau 82.5%. The results of the volumetric measurements together with results of the proteins in the CSF did not increase the sensitivity (88.9%) but increased specificity to 96.8% and the percentage of correct classification to 95%. Sensitivity, specificity and the percentage of correct classification for parameters which were statistically significant are presented in Table IV.

Discussion

The obtained results confirm that the use of volumetric assessment of selected brain structures and the assessment of Aβ and tau protein in CSF can be useful in predicting the MCI progression to AD. However, the biggest limitation of our study was the small group of patients (40 persons), so the results are limited. Surprisingly, sensitivity for volumetric measurements was almost 90%, whereas in our previous study (101 patients diagnosed with MCI) we have obtained sensitivity of 64.7%, specificity of 96.4% and classification rate of 91% (in this study 90%) [11]. Similar results using volumetry were presented by Convit. His study group was also limited (46 patients); sensitivity of the prediction of conversion by using volume of hippocampus was 57% (in our study 66.7%) and by using all measured volumes increased to 93% (in our study to 88.9%), specificity was 97% (in our study 90.3%) [3]. Taking into account individual volumetric measurements the results obtained in our previous study were confirmed, i.e. the highest sensitivity was for the hippocampus and then for the left entorhinal cortex [11]. Our results are contrary to the results presented by Dickerson (23 patients diagnosed with MCI, observation period of 12-77 months) or Stoub (23 patients diagnosed with MCI and 35 from the control group, observation period was 5 years) in whose studies volume of

Table IV. Sensitivity, specificity and classification rate for single parameters

	Sensitivity (%)	Specificity (%)	Correct classification rate (%)
LH	66.7	77.4	75
RH	66.7	74.2	72.5
LERC	55.6	67.7	65
RITG	55.6	64.5	62.5
Aβ	77.8	64.5	67.5
Total tau	66.7	83.9	80

LH – left hippocampus, RH – right hippocampus, LERC – left entorhinal cortex, RITG – right inferior temporal gyrus

entorhinal cortex was a better parameter than volume of hippocampus in predicting MCI conversion to AD [4,13]. It should be noted that higher sensitivity compared to single volumetric measurements, was obtained for Aβ (77.8%), as in Egli's study, and as for total tau it was the same as for hippocampi (66.7%) but total tau has had higher specificity compared with Aβ and hippocampi which gives the best percentage of correct classification (conversion vs. no conversion) for total tau protein (80%). Specificity increased after using a few parameters together [5].

Biomarkers were also studied in Ewers' study; the most sensitive parameter was volumetric measurement of left hippocampus and the highest percentage of correct classification was achieved by using the right entorhinal cortex volume. Sensitivity and specificity of prediction of MCI conversion to AD increased in the models using parameters of cerebrospinal fluid [6].

The study which used ADNI database [14] on 162 patients with diagnosed MCI showed superiority of the biomarkers from CSF in predicting the conversion of MCI to AD (sensitivity 76.4% vs. 65.4%), the percentage of correct classification for both markers was the same (65.4%) but increased (to 68.5%) using both methods together (follow-up period of 36 months).

In Prestia's study the highest sensitivity was for Aβ (79%) as a single biomarker, which was also confirmed in our work, with the highest specificity for the volumetric measurement of hippocampus (76%). The study group consisted of 103 patients diagnosed with MCI (from two databases: ADNI and TOMC and follow-up period was 36 ± 12 months) [12].

The follow-up period for our study was 2 years and there is a possibility that in the coming years progression to AD in subsequent patients can be observed, so the proportion of converters to non-converters can change and sensitivity of used methods can also improve. The patients enrolled in our study met the MCI criteria [16]; conversion to AD was diagnosed in the patients who progressed to dementia and met criteria for probable AD [9] but even in such a small group there is a probability of a mistake in diagnosis (other type of dementia for example FTD, DLB).

Conclusions

The above-mentioned biomarkers seem to be important parameters, in particular when biochemical biomarkers are used together with volumetric ones. Possibility of CSF analysis with Aβ and tau protein assessment is nowadays easier. MRI is also widely available. Confirmation of effectiveness of the method requires the study and observation on a larger group of patients with diagnosed MCI.

Disclosure

Authors report no conflict of interest.

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Original paper

Folia Neuropathologica

Disturbed integrin expression in the vascular media in CADASIL

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Folia Neuropathol 2016; 54 (4): 375-381 DOI: 10.5114/fn.2016.64816

Abstract

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is an inherited angiopathy characterized by degeneration and loss of vascular smooth muscle cells (VSMCs) of still unknown pathomechanism. Many functions of VSMCs, such as adhesion, apoptosis, contraction, differentiation, migration, and proliferation are determined by integrins – surface adhesion receptors involved in binding and interactions between cells and extracellular matrix (ECM). Since integrins play such an important role in VSMCs biology, disturbances in their expression may influence myocytes behavior and fate in CADASIL. In this study, we focused on the most important compounds of VSMCs integrins: subunits α_4, β1, and β_3 in an attempt to elucidate their immune expression in the arterial media of CADASIL patients. The immunohistochemistry revealed a decreased expression of integrin β1 subunit ($p < 0.001$) but similar to the control expression of integrin subunits α_4 and β_3. Decreased β1 immunoreactivity was observed in capillary vessels, arterioles, and small arteries. The abnormal immune expression of integrin β1 subunit was found even in microvessels without microscopically noted degenerative changes, which suggests that this is an early phenomenon in CADASIL. Since integrin β1 subunit is a compound of 10 heterodimer integrin receptors, its disturbed expression may significantly influence VSMCs biology leading to myocytes degeneration and loss via anoikis – a type of apoptotic cell death due to loss or inappropriate cell adhesion to ECM.

Key words: *CADASIL, integrin, microangiopathy, tunica media, vascular smooth muscle.*

Introduction

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is an inherited angiopathy due to *NOTCH3* mutations and it is characterized by degeneration and loss of vascular smooth muscle cells (VSMCs) and pericytes. In spite of intensive research studies, the relationship between *NOTCH3* defects and the pathomechanism of destruction of small blood vessels in CADASIL is still unknown.

VSMCs are cells with a high degree of plasticity which can undergo reversible phenotypic changes in response to environmental stress and vascular injury. Disturbances both in interactions between vessel wall cells and extracellular matrix (ECM) as well as in intercellular communication play an important role in control of VSMCs behavior. This exchange of information occurs through specialized types of cell surface receptors: mainly integrin receptors but also discoidin domain receptors, cell

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surface proteoglycan receptors, and the hyaluronan receptor CD44a [25].

According to their name, integrins integrate ECM with cells and cells together. An integrin receptor is composed of two subunits: α and β. Subunit α is responsible for the receptor binding with ligand and determines integrin specificity while subunit β takes part in interaction with cytoskeletal proteins and determines the receptor function [5]. In cerebral blood vessels, integrins are also involved in many other important functions such as:

- mechanotransduction – a process of converting mechanical stimuli such as press or stretching to intracellular signals resulting in a specific gene expression,
- regulation of vasomotor response: contraction (integrins α1β1 and α5β1) and relaxation (integrin αvβ3) of the vessel [15],
- regulation of vessel permeability by modification of cadherins in endothelial adherent junctions and by contraction of collagen fibers in ECM (integrin α2β1) [20],
- regulation of VSMCs tensegrity (resting tension in VSMC triggered by external mechanical forces, which influences myocyte behavior) [17],
- activation of the cell death program [24]; integrins belong to a group of dependence receptors which lack of activity can lead to cell apoptosis,
- modulation of cells shape by linkage with cytoskeletal actin and reorganization of actin filaments.

In VSMCs, there are 13 combinations of integrins: α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α8β1, αVβ1, αVβ3, αVβ5, α6β4 [15,16]. Some of them are present in physiologic conditions or on specific myocyte phenotype while others are present only in pathology. On VSMCs, the most abundant integrin subunit is β1 subunit which is a compound of 10 heterodimeric receptors.

Since one of the possible causes of VSMCs damage in CADASIL may be a disturbed expression of integrins, we examined in vessels, an immune expression of selected integrins with a key importance for VSMCs biology and fate.

Material and methods

The study was performed on tissue samples from 10 autopsy brains and 10 skin-muscle biopsies of 20 different patients with CADASIL (Table I). The diagnosis was established on the basis of ultrastructural examination in which deposits of granular osmiophilic material (GOM) in the vessel wall [6] (pathognomonic for the disease) were found. In majority of the cases, genetic tests additionally confirmed CADASIL. Aged-matched control material was composed of 5 normal human brains and 5 normal skin-muscle biopsies. Histopathological and immunohistochemical examinations were performed on formalin-fixed and paraffin embedded sections. On tissue slides immune reactions with antibodies against integrin subunits: β1 (Novocastra, NCL-CD29, 1 : 40), α_4 (Santa Cruz Biotechnology, sc-14008, 1 : 50) and β_3 (Santa Cruz Biotechnology, sc-52589, 1 : 100) were applied. The immunochemistry tests were performed according to a routine streptavidin-biotin-peroxidase method with the using of DAKO LSAB 2 SYSTEM (DAKO, K0675), developed by diaminobenzidine, and assessed by light microscopy.

Intensity of the immune reactions was measured using ImageJ software and gray levels method. In digital photos of slides, color was converted into the gray scale and then intensities of the gray values were measured across the entire vessel wall. Results of the measurements were statistically analyzed with the use of t-Student test for independent variables.

Results

In CADASIL, preserved VSMCs visible on transverse sections through small arterial vessels often showed around shape with a perinuclear halo (Fig. 1). Such cell appearance is sometimes called "fried egg" look. In small arteries, the round-shaped myocytes were localized mainly in the peripheral part of the media while in arterioles they were present through the whole media thickness.

In CADASIL, immunohistochemistry revealed a decreased expression of the integrin subunit β1 in the vascular media in comparison to control material ($p < 0.001$, CADASIL: SD 39.6 × 10^6; mean value 61.7 × 10^6, control: SD 30.1 × 10^6; mean value 94.2 × 10^6) (Fig. 2A). Decreased immunoreactivity to integrin β1 was observed in small arteries and arterioles independently of whether the examined vessels revealed or not microscopically noted degenerative changes (Fig. 2D, F). Also capillary vessels (Fig. 2B, C) and some venules showed a diminished expression of integrin β1. In arterial vessels with moderately advanced degenerative changes, the expression of integrin β1 within tunica media was diminished but still present, and revealed an irregular patchy char-

Table I. Material – clinical data

No.	Sex/Age	Clinical data			
		Autopsy material			
1	F/73	At 68 yo ischemic stroke with left hemiparesis; at 70 yo hemorrhage into the left parieto-occipital area			
2	M/58	Progressing dementia of unknown onset, recurrent ischemic strokes			
3	M/64	At 51 yo ischemic stroke with left hemiparesis; later recurrent bilateral ischemic strokes			
4	F/52	Since 40 yo progressing dementia, epilepsy, confirmed NOTCH3 mutation			
5	M/59	Since 53 yo progressing dementia			
6	M/45	Since 40 yo progressing dementia, confirmed NOTCH3 mutation			
7	M/38	Since 33 yo progressing dementia, epilepsy, positive family history			
8	F/45	At 39 yo right hemiparesis with aphasia, later recurrent ischemic strokes, progressing dementia, positive family history – daughter of patient no. 7			
9	F/56	Since 44 yo cognitive disturbances; later recurrent ischemic strokes, progressing dementia; negative family history			
10	F/58	Migraine, since 48 yo recurrent strokes, confirmed NOTCH3 mutation			
		Biopsy material			
1	F/51	Since 41 yo progressing dementia, 2 ischemic strokes with right hemiparesis and aphasia; positive family history, confirmed NOTCH3 mutation			
2	M/44	At 42 yo ischemic stroke with left hemiparesis; since 43 yo – progressing dementia, positive family history			
3	M/53	Migraine, since 50 yo progressing dementia, unknown family history			
4	F/35	At 35 yo two seizures attacks, hyperintense changes in cerebral white matter in MRI, positive family history, confirmed NOTCH3 mutation			
5	M/61	At 44 yo ischemic stroke, later 2 bilateral ischemic strokes, hyperintense changes in the cerebral white matter in MRI; positive family history – father of patient no. 4, confirmed NOTCH3 mutation			
6	M/39	At 33 yo TIA with left hemiparesis and aphasia, normal brain MRI, positive family history – brother of patient no. 4, confirmed NOTCH3 mutation			
7	F/57	Migraine, hyperintense changes in cerebral white matter in MRI, negative family history			
8	F/42	Since 42 yo recurrent TIA, hyperintense changes in the cerebral white matter in MRI, negative family history, confirmed NOTCH3 mutation			
9	M/58	Since 57 yo cognitive disturbances, negative family history, negative genetic test (examined only 3 NOTCH3 exons)			
10	M/44	At 42 yo and 45 yo ischemic strokes with left hemiparesis, positive family history, confirmed NOTCH3 mutation			
		Control material			
No.	Sex/Age	Clinical data	No.	Sex/Age	Clinical data
		Autopsy material			Biopsy material
1	F/64	Ischemic brainstem stroke	1	M/41	Myopathy suspicion
2	M/59	Ischemic stroke	2	F/45	Collagenosis suspicion
3	M/57	Pancreas carcinoma	3	F/46	Myopathy suspicion
4	F/43	Myeloma	4	M/42	Vasculitis suspicion
5	F/32	Leukemia	5	F/39	Vasculitis suspicion

acter (Fig. 2F, H, I). It is interesting that in many vessels the immunolabel to integrin β1 was better preserved in more luminal than peripheral part of the media (Fig. 2H, I). Frequently, pericellular immune reaction to integrin β1 was negative even in microscopically normal but peripherally localized myocytes (Fig. 2H, I). Arterial vessels at a very advanced stage of degeneration demonstrated a loss of or evidently decreased immunoreactivity to integrin β1 in the media (Fig. 2G).

Immunolabels to integrin subunits α_4 and β_3 were weak and similar in CADASIL and controls.

In CADASIL, the above changes in an integrin expression were similar in cerebral, skin and skeletal muscle vessels. That is why statistics were performed together for autopsy and biopsy material.

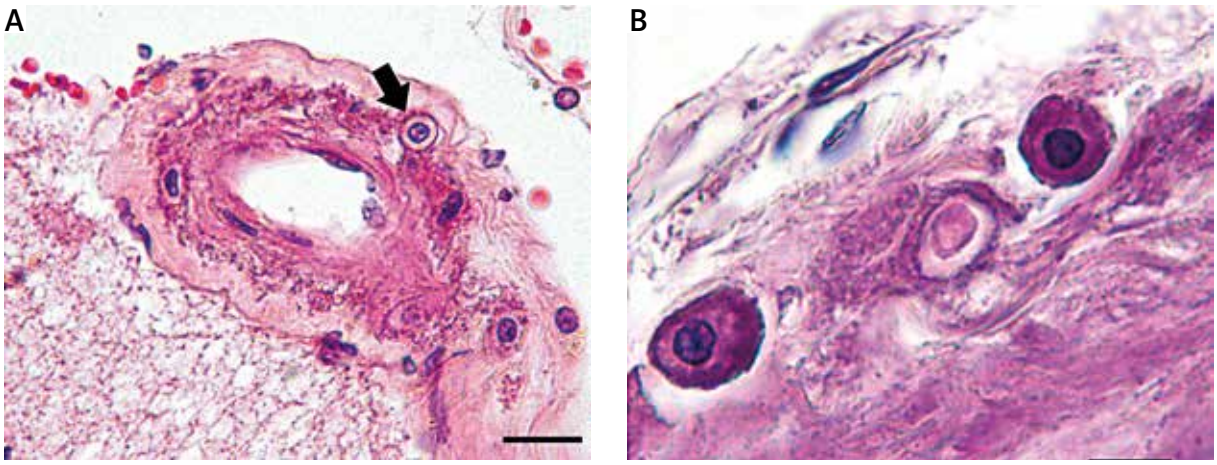

Fig. 1. Rounded vascular smooth muscle cells (VSMC) in the vessel wall in CADASIL; H&E. **A**) Meningeal arteriole with basophilic granular degeneration. A single round myocyte with a perinuclear hallo ("fried egg" appearance – arrow) in the vascular media is visible; bar: 50 µm. **B**) Fragment of the vascular media with three myocytes. Severely damaged middle myocyte with pale cytoplasm is surrounded by basophilic granular material while two dark cells on its sides are round with clear (empty?) pericellular area; bar: 20 µm.

Discussion

One of the characteristic morphological changes observed by us in CADASIL was a round shape of myocytes in the tunica media. This phenomenon has been visible on some figures included in already published CADASIL papers but, surprisingly, it has not received any attention of the authors. In small arteries and arterioles, the media are composed of a layer of smooth muscle cells with circular arrangement. Therefore in normal conditions, on transverse sections through the vessel wall VSMCs have a longitudinal shape. But in CADASIL preserved myocytes were often rounded. Such rounded appearance is characteristic for cells detached from ECM and devoid of any physical influences from the surrounding environment [10]. The cell shape is controlled by the cytoskeleton, its connections with integrins and continuous tension (tensegrity) being a reaction of the cell to applied pressure and interactions with external substrates. Loss of tensegrity leads to cell retraction and probably such phenomenon was observed by us in the CADASIL vascular media.

In the examined CADASIL microvessels, a decreased immune expression of integrin subunit β1 in the media was found. The diminished immunolabel to integrin β1 was noted even in microscopically normal vessels, which suggests that this alternation is an early change in CADASIL.

In VSMCs, the β1 subunit is a compound of 10 different heterodimer integrin receptors, hence it is not possible to define whether its abnormal immune expression, as observed by us, is related to one or more types of the integrin receptors. Moreover, an individual integrin may bind to different components of ECM just as a single matrix component may bind to different integrins. Taking into account this complexity of interactions it is not surprising that integrin-mediated cellular responses are quite varied and multifaceted. Therefore it is difficult to discuss specific consequences of the diminished expression of integrin β1 in CADASIL but only its general impact.

CADASIL is due to mutations in the *NOTCH3* gene. Integrins constitute a crucial element of the Notch signaling system but the relationship is mutual. On the one hand, transfer of the intracellular domain of the Notch 3 receptor to the cell nucleus leads to unlocking of genes transcription and integrins activation [19]. On the other hand, integrins play an important role in regulation of the Notch 3 receptor activity by mediation of its caveolin-1-dependent endocytosis [7]. Internalization of caveolin-1 inhibits Notch 3 endocytosis and may favor accumulation of the receptor on the surface of VSMCs – the other than GOM deposits morphologic change characteristic for CADASIL.

Integrin β1 engagement also results in ligand-independent activation of many different receptors,

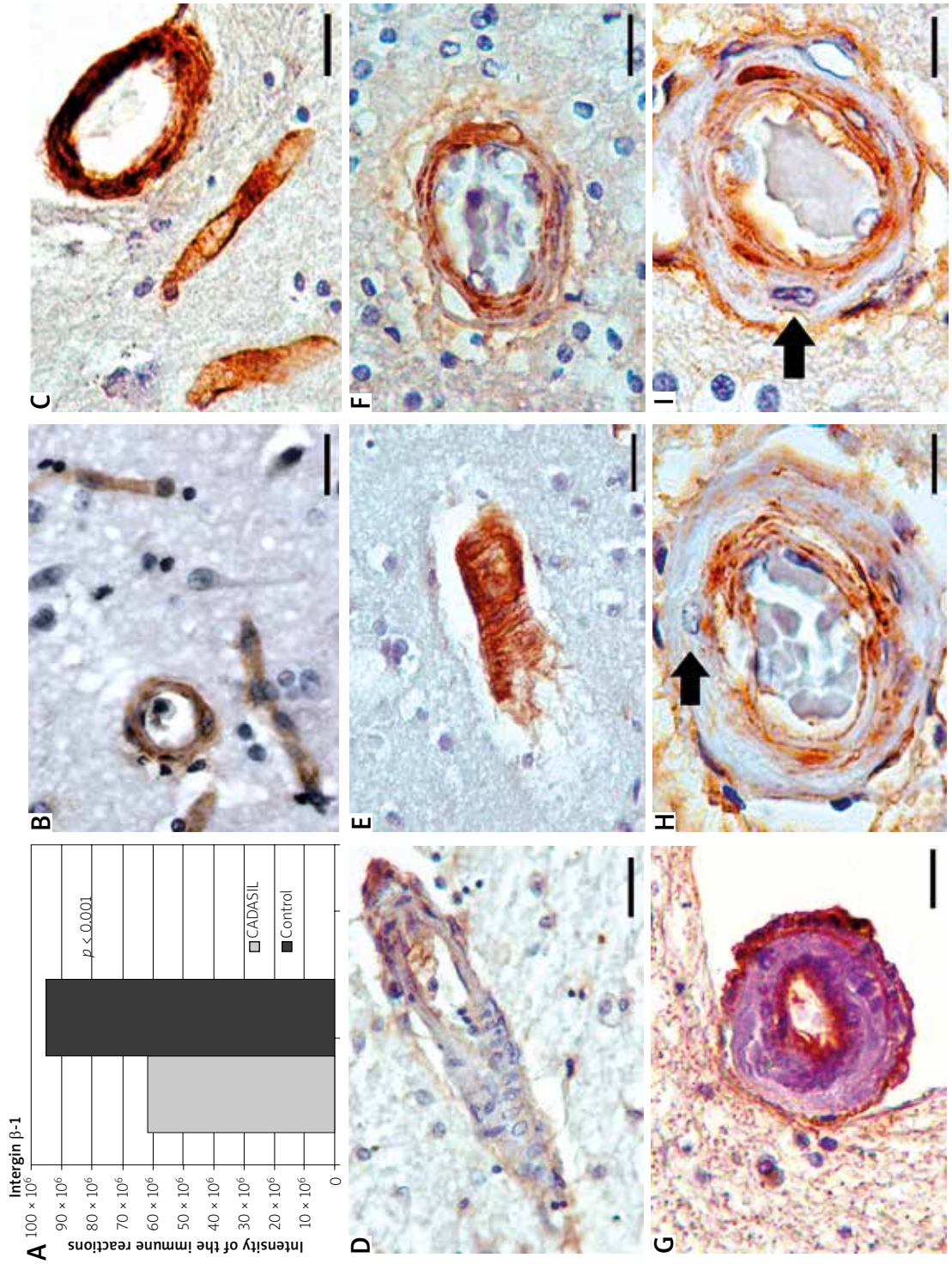

Fig. 2. Immune expression of integrin subunit β1 in various sized cerebral vessels in CADASIL and control materials. **A**) Results of the statistical analysis demonstrate the significant difference ($p < 0.001$) in the intensity of the immunolabel to integrin subunit β1 in CADASIL and control cases. **B-E**) Diminished immune expression of integrin β1 in parenchymal small arteries, arterioles and capillary vessels in CADASIL (**B**,**D**) and controls (**C**,**E**); bars: 100 µm. **F**) Structurally normal white matter arteriole with a diminished expression of integrin β1, bar: 50 µm. **G**) Small meningeal artery at the advanced stage of degeneration. Negative immunoreactivity to integrin β1 in the thickened tunica media and positive immunolabel of the adventitia and endothelium; bar: 100 µm. **H**, **I**) Irregular, patchy character of the integrin β1 immune expression in small white matter arterioles. Single relatively well preserved vascular smooth muscle cells (VSMC) with immunonegative pericellular reaction (arrows) in the peripheral part of the tunica media are visible; bars: 50 µm.

among them epidermal growth factor receptor (EGFR) [18]. In detached cells disengagement of integrin β1 leads to down-regulation of EGFR expression culminating in upregulation of death signaling [22]. In the literature, we have not found any information concerning EGFR expression on VSMCs in CADASIL. But mutations in the *NOTCH3* gene responsible for development of the disease affect only that part of the Notch 3 receptor which contains EGF repeats motif. Although EGF-like motif is shared by many proteins of diverse functions and its significance is not well defined, one of its functions is mediation of adhesive interactions [22]. Therefore an abnormal integrin β1 expression in CADASIL arteries may not only disturb, per se, myocyte adhesion to ECM but also it may have an influence on adhesive interactions mediated by the Notch 3 receptor.

Integrins are involved in regulation of cell viability through their interaction with ECM. They sense mechanical forces arising from contacts with ECM and convert them into intracellular signals leading to gene expression. It was reported that in blood vessels signals undergoing from integrin β1 protect VSMCs from apoptosis due to mechanical stress [29]. Moreover, integrins 1β1, α2β1, α3β1, α5β1, α6β1 (presented on VSMCs surface) activate pro-survival pathways [2,8]. One of the integrin-mediated survival signaling pathways occurs through the caveolin-1-mediated binding of integrins to the adaptor protein (protein Shc). This type of integrin binding allows the cell to escape from death by a special type of apoptosis called anoikis [3,28]. Anoikis, a Greek word meaning loss of home or homelessness, is due to loss or inappropriate cell adhesion to ECM. Anoikis is observed both physiologically (in normal skin, in colonic epithelium, in the involuting mammillary glands), as well as in many pathologic conditions such as metastatic spreading and cardiovascular degenerative processes including VSMCs disappearance in aneurysms and varicose veins, extensive loss of vascular cells during cardiovascular infections, cardiac myocyte detachment in heart failure, and plaque rupture in atherosclerosis [4,12,27].

It was demonstrated that in cells undergoing apoptosis the expression of integrin β1 is diminished and is about 65% [14]. A decreased or absent integrin β1 expression on VSMCs not only inhibits pro-survival pathways and facilitates their death but can also change the phenotype of myocytes towards synthetic one which is more sensitive to apoptosis [1].

Investigations on the integrin expression on blood vessels showed that a decreased β1 expression can disturb vasomotor reactivity [15,16]. In experimental investigations, impaired cerebral vasoreactivity in a transgenic mouse model of CADASIL was observed [13]. Also clinical studies on CADASIL patients demonstrated abnormal vasoreactivity [9,26] and a reduced cerebral blood flow [20,23] in the course of the disease. Integrin subunit β1 as a part of α1β1 and α5β1 integrin receptors is involved in vasoconstriction and transfer of signals originating from ECM and regulating permeability of calcium channels on VSMCs and myocytic voltage value. For these reasons abnormal immune expression of integrin subunit β1 as observed by us may result in impaired vasoreactivity. In CADASIL, abnormal vessel mechanoresponse precedes development of morphological changes in vessels and white matter lesion both in patients and in animals [11]. These clinical and experimental observations reinforce our hypothesis that an abnormal integrin β1 expression in microvessels is an early phenomenon in CADASIL.

Putting all the data together, we suggest that disturbed expression of integrin subunit β1 in CADASIL can be an important part of the disease pathomechanism leading to improper activity of the Notch 3 signaling pathway and VSMCs degeneration and loss via anoikis.

Disclosure

Authors report no conflict of interest.

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Original paper

Folia Neuropathologica

Protective role of dexmedetomidine in unmethylated CpG-induced inflammation responses in BV2 microglia cells

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Folia Neuropathol 2016; 54 (4): 382-391 DOI: 10.5114/fn.2016.64817

Abstract

Unmethylated CpG DNA, as a stimulatory molecule, has potent pro-inflammatory effects in the central nervous system (CNS). Dexmedetomidine (DEX) has been confirmed to exert anti-inflammatory effects in CNS. Our study was aimed to explore the effects of DEX on tumor necrosis factor-α (TNF-α) expression in unmethylated CpG DNA-challenged microglia. In vivo, after 3 d intracisternal injection of ODN1668, we evaluated the severity of meningitis with or without DEX via pathobiology method and detected the expression of TNF-α from molecular and protein levels. In vitro, we explored whether the ODN1668 could activate microglia to express TNF-α and the inhibition mechanism of DEX. Our results demonstrated that DEX could alleviate the severity of ODN1668-induced meningitis. And while BV2 microglia was stimulated by ODN1668 for different time, TNF-α was increased in mRNA and protein levels but the effect was attenuated by DEX via decreasing phosphorylated AKT and ERK.

Key words: *DEX, unmethylated CpG DNA, microglia.*

Introduction

Unmethylated CpG motifs in bacterial DNA exert stimulatory effects on murine and human lymphocytes to secret interleukin-6 (IL-6), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [8,26,27,33,55]. Synthetic oligodeoxynucleotides with immunostimulatory CpG motifs also have these effects [52]. Toll like receptor-9 (TLR-9) is essential for CpG DNA to activate innate immune response [23]. It has been proven that bacterial DNA could cause many diseases, such as arthritis [11], septic shock [46], meningitis [12] and skin inflammation [32]. In the central nervous system, unmethylated CpG DNA could activate microglia and astrocytes to express TNF-α, IL-12 and NO [9,48], and it is believed that exacerbation of meningitis caused by increased levels of these inflammation cytokines leads to the damage of the blood-brain barrier (BBB) [39,40].

Tumor necrosis factor α is a homotrimeric transmembrane protein that plays an important role in innate immune defense and maintenance of homeostasis at the cellular, tissue and organ levels [49]. Excessive TNF-α in the central nervous system has been verified in patients and animal models of a wide range of CNS pathologies such as Alzheimer's disease (AD) [17], Parkinson's disease (PD) [4], multiple sclerosis (MS) [24] and meningitis [29]. In the animal model, circulating TNF-α could cross the BBB into brain parenchyma by a special saturable transport system [21]. Inflammatory stimuli such as

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LPS [58], CpG DNA [9], cytokines could induce TNF-α production by additional CNS cell types, especially microglia, which is recognized as an abundant source of TNF-α [43]. In the CNS inflammatory diseases, TNF-α could induce leukocyte adhesion to cerebral vessels through upregulating expression of adhesion molecules such as VCAM and ICAM [37], and also could damage BBB [10]. Oligodendrocyte apoptosis and neuroinflammation could be induced by overexpression of TNF-α from glia [38].

Microglia, resident macrophages in the CNS, are sensitive to and activated by trauma, neurodegenerative diseases and infection [36]. Apoptosis of dopaminergic neurons could be mediated by LPS-activated microglia [14]. The pro-inflammation cytokines secreted by microglia are IL-1, IL-6, IL-10, IL-12, TNF-α, TGF-β and chemokines [41]. Microglia play distinctive roles in different diseases, it has been reported that microglia have a neuroprotective role in Alzheimer's disease [28].

Dexmedetomidine (DEX), a highly selective and potent α2-adrenoreceptor agonist, provides excellent sedation, analgesic and anxiolytic effects [3]. A lot of evidence has shown that DEX exerts anti-inflammatory effects [54]. DEX could ameliorate intestinal injury induced by CLP [7] and attenuate LPS-induced lung injury [53]. However, the mechanisms of neuroprotective effects of DEX in microglia activation have not been elucidated. Thus, the purpose of this study was to evaluate the effects of DEX on CpG DNA-induced microglia activation and illuminate possible mechanisms of its neuroprotective actions.

Material and methods

Animals

Male C57BL/6 mice (6-7w) were obtained from the Nanjing University Animal Center. All experimental procedures were conducted with the approval of the Ethics Committee of Nanjing Medical University and in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health publication No. 85-23, National Academy Press, Washington DC, revised 1996).

ODN1668 and ODN1720

Oligonucleotides 1668 and 1720 were synthesized by Invitrogen™.1668, 5'-TCC ATG ACG TTC CTG ATG CT-3'; 1720, 5'-TCC ATG AGC TTC CTG ATG CT-3'.

Injection protocol

Mice were anesthetized by chloral hydrate, after shaving and disinfection of the injection area, 10 µl of ODN was injected intracisternally into mice. Three days after injection, mice were sacrificed and brains were collected.

HE staining and immunohistochemical staining

Brain tissues were fixed in formalin and dehydrated by concentration gradient of ethanol and embedded into paraffin, and then were transversely cut into 5 µm sections, which were subjected to be stained with hematoxylin and eosin. The brain sections were dewaxed by Xylene, then hydrated, after heat antigen retrieval, the sections were labeled with anti-TNF-α antibody (R&D), biotin-conjugated goat anti-rabbit (KPL) as the secondary antibody labeled the primary antibody and combined with Elite AB (Vector), at last DAB were used for color development.

RNA extraction and real-time PCR

Total RNA was extracted from brain tissues and cells using Trizol Reagent (Takara) according to the manufacturer's instructions. The concentration of RNA was confirmed using Ultra Micro-ultraviolet spectrophotometer (One drop OD1000, China). All of RNA was reverse-transcribed using the PrimeScript® RT reagent Kit (TaKaRa) according to the manufacturer's instructions. Real-time PCR using Takara (Takara) was carried out on the 7300 System (ABI) for the detection of PCR products.

TNF-α reverse: 5'-ACATTCGAGGCTCCAGTGAATTCGG-3';
TNF-α forward: 5'-GGCAGGTCTACTTTGGAGGTCATTGC-3'.

Western-blot

RIPA was used for cells lysis, and then the extracted protein concentration was determined with BCA methods. The protein samples were loaded into 10% SDS-PAGE and transferred to the PVDF membrane (polyvinylidene fluoride), then the PVDF membrane was incubated with anti-p-JNK, JNK, p-AKT, AKT, p-ERK and ERK antibodies respectively overnight at 4°C, next the PVDF membrane was incubated with anti-rabbit antibodies conjugated with HRP, immunocomplex was detected by the enhanced horseradish peroxidase/luminal chemiluminescence system (ECL).

Cell Proliferation Assay

Cell counting Kit-8 (CCK-8) was applied to cell proliferation. The BV2 cells were cultured in triplicate in 96-well plates treated by 0.5 μM, 1 μM, 5 μM and 10 μM DEX for 6 h, then cell proliferation was determined according to the manufacturer's instructions (Beyotime, China).

Cytokine Bead Array

The Cytokine Bead Array was used for measurement of TNF-α in the supernatants of BV2 cell stimulated by 1668ODN or control. 50 μl of each sample was mixed with 1 μl of TNF-α capture beads for 1 h at room temperature then added into 1 ul of PE detection reagent for 1 h at room temperature in the dark. Beads were washed with wash buffer and centrifuged at 200 g × 5 min, discarded the supernatants. The pellets were resuspended with 200 μl of wash buffer and assayed on FACS Calibur (BD Biosciences). The concentration of TNF-α was determined by the software provided.

Immunofluorescence

The stimulated BV2 cells were fixed with 2% paraformaldehyde for 10 min at room temperature, the primary antibodies Iba-1 (Wako) and TNF-α (R&D) co-incubated with BV2 cells at 4°C overnight, then the secondary antibodies, anti-rabbit Alexa Fluor 488 and anti-goat Alexa Fluor 584 co-incubated with BV2 cells at room temperature for 1 h, DAPI was used for labeling nucleus. The images were obtained with Zeiss microscope.

Isolation of primary microglia

Microglia of newborn mice were prepared as described [50]. Briefly, brains of newborn C57BL/6 mice were dissected and dissociated. The cells were seeded in DMEM-F12 medium containing 10% FBS with 75 cm^2 culture flask. On day 14, cultures were agitated on a rotary shaker at 240 rpm for 3 h at 37°C. Microglia were collected from the supernatant.

Assessment of severity of meningitis

The severity of meningitis was scored by a predetermined scheme: score 0 – no meningitis; score 1 – occasional occurrence of inflammatory cells; score 2 – inflammatory cells forming an infiltrate not involving the entire depth of the subarachnoid space; score 3 – inflammatory infiltrate involving the entire subarachnoid space.

Statistical analysis

All data were analyzed as mean and standard deviation (mean ± SD). Student's *t*-test was used to compare differences followed by paired comparisons. A value of $p \leq 0.05$ was regarded as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results

Intracisternal injection of ODN1668 could cause meningitis and activate microglia

It has been reported that the intracisternal injection of ODN1668 could cause meningitis [10], so ODN1668 was employed in our study, ODN1720 as a control, which contained methylated CpG sequences. The results showed that the mice treated with ODN1668 developed meningitis, but not controls (Fig. 1A). The scores of severity of meningitis were much higher compared with controls (Fig. 1B). It was known that TNF-α played an important role in bacterial meningitis, and the levels TNF-α were obviously elevated in the cerebrospinal fluid [1,18]. Similarly, the representative immunohistochemical photographs exhibited an abundant TNF-α expression in the cortex of ODN1668-treated mice (Fig. 1C). To measure the mRNA level of TNF-α in brain tissues, ODN1668-treated brain tissues were analyzed by real-time PCR, and the result showed an obvious increase in TNF-α in ODN1668-treated mice compared with controls (Fig. 1D).

ODN1668 induced BV2 to express TNF-α

We have proved that there was plenty of TNF-α expression in the brain which were administered ODN1668 intracisternally. As mentioned above, microglia were the major resource of TNF-α in CNS [23], so we tested whether ODN1668 could induce microglia to express TNF-α *in vitro*. Incubation of BV2 cells with ODN1668 for 12 h stimulated the TNF-α production detected by immunofluorescence (Fig. 2A). Furthermore, real-time PCR showed that ODN1668 time-dependently increased the TNF-α mRNA levels and peaked 6 h after stimulation (Fig. 2B). It has been

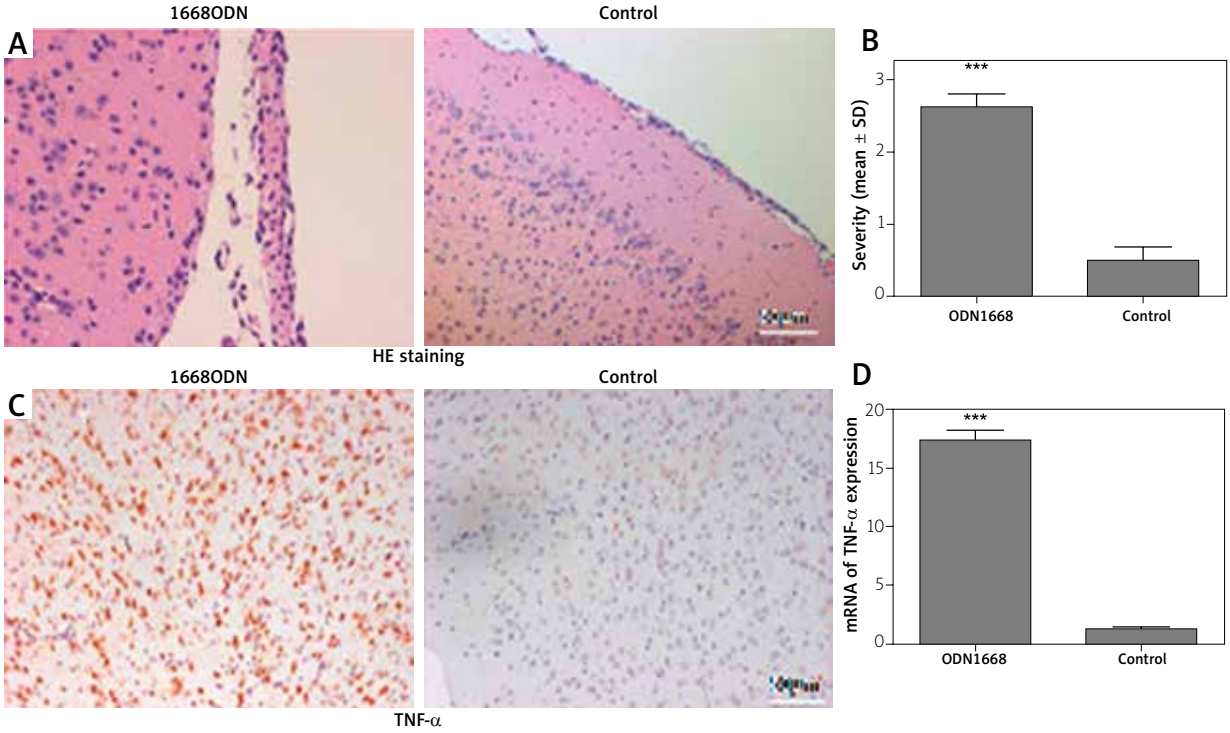

Fig. 1. Intracisternal injection of ODN1668 can activate microglia. **A**) Histopathology of the brain of a C57BL/6 mouse killed 3 d after intracisternal inoculation of ODN1668 and ODN1720 (1OD) (n = 5, magnification ×200). **B**) Score of meningitis severity in C57BL/6 mice treated with ODN1668 for 3 d (1OD per mouse, n = 5). Values are the mean ± SD score of inflammatory cell infiltrate score. ***p < 0.001 compared with the control group. **C**) Immunohistochemical analysis of TNF-α in the brain 3 days after intracisternal inoculation of ODN1668 and ODN1720 (1OD) (n = 5, magnification ×200). **D**) Three days after intracisternal injection of ODN1668, brain tissues were obtained and analyzed by real-time PCR, ODN1720 as control. ***p < 0.001 compared with the control group.

reported that microglia could be activated via MAPK pathway [56], so we developed a question whether ODN1668 could activate microglia via this path. To answer it, we stimulated BV2 with ODN1668, western blot results suggested that ODN1668 significantly increased AKT, ERK and JNK phosphorylation and after stimulation the levels of phosphorylation gradually increased, which p-AKT and p-ERK peaked at 20 min and p-JNK peaked at 40 min (Fig. 2C, D).

Dexmedetomidine could inhibit microglia to express TNF-α

CCK-8 assay was used to evaluate to the toxic effects of DEX on BV2. BV2 cells were incubated with different concentrations of DEX (0.5 μM, 1 μM, 5 μM, 10 μM) for 6 h. The results indicated that DEX exerted no obvious toxic effects on BV2 (Fig. 3A). To test whether DEX could depress ODN1668-induced microglia inflammatory response, BV2 cells were pretreated with different concentrations of DEX for 30 min [57], followed by the addition of ODN1668. Real-time PCR results showed that DEX significantly inhibited TNF-α expression in a dose-dependent manner (Fig. 3B). In addition, the primary microglia were isolated from mice and pre-incubated with DEX for 30 min before ODN1668 stimulation for another 6 h. The fluorescence images demonstrated that DEX remarkably inhibited the TNF-α expression in response to ODN1668 (Fig. 3C). Then, we examined whether DEX inhibits TNF-α via the MAPK pathway. Western-blot results demonstrated that DEX inhibited phosphorylated-AKT and ERK levels induced by ODN1668 (Fig. 3D, E).

Injection of dexmedetomidine reduced the generation of TNF-α *in vivo*

To know whether DEX could treat ODN1668-caused meningitis *in vivo*, mice received DEX (50 μg/kg, *i.c.v.*) 30 min before ODN1668 treatment (1OD, *i.c.v.*), compared with single treatment with ODN1668

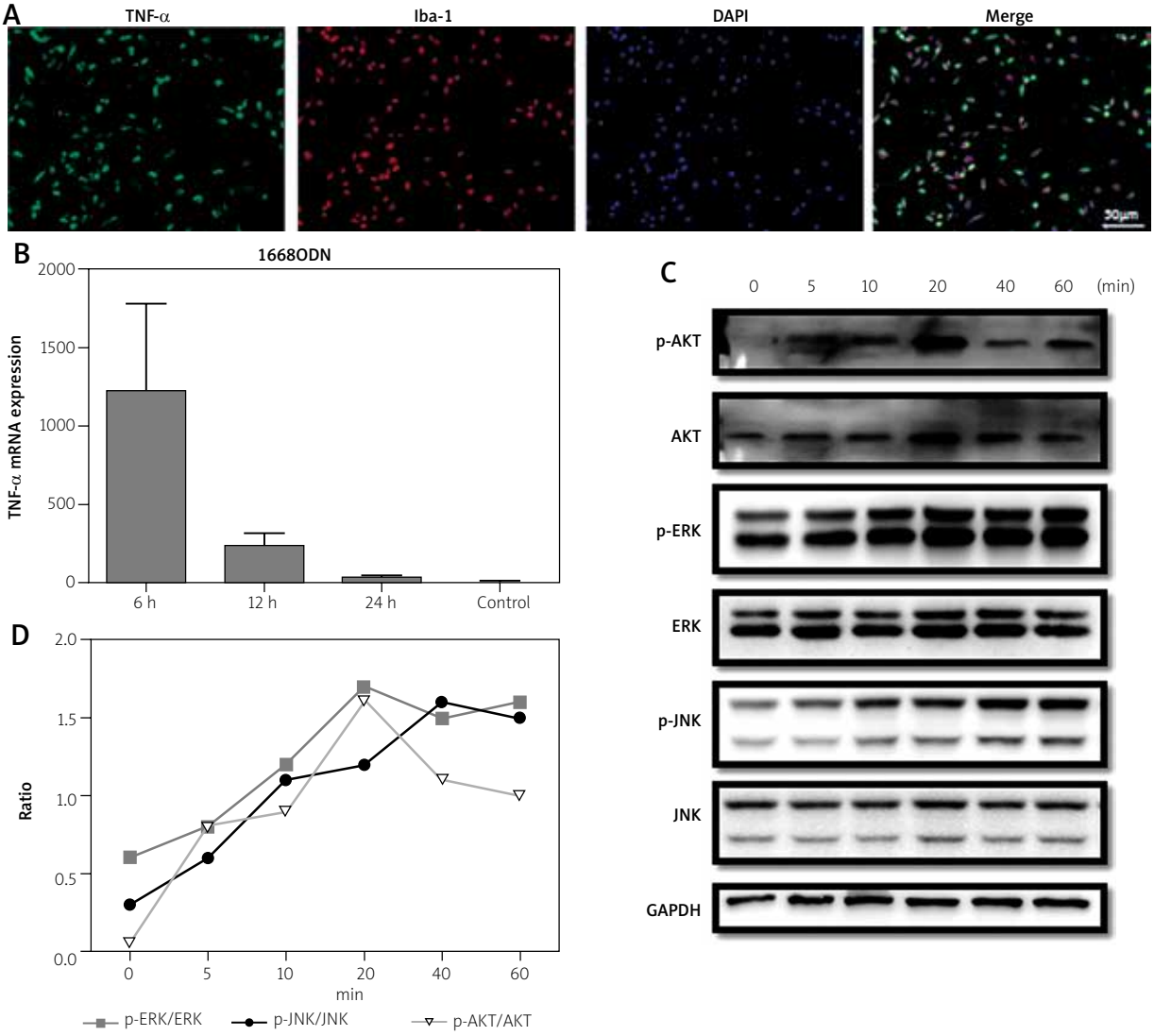

Fig. 2. ODN1668 could induce BV2 strain to express TNF-α. **A**) Representative images of immunofluorescence staining with primary anti-Iba-1 and anti-TNF-α antibodies. Immunoreactivities for Iba-1 (a marker of microglia, red) or TNF-α (green) were visualized by Alexa Fluor 488-conjugated donkey anti-goat IgG and Alexa Fluor 584-conjugated donkey anti-rabbit IgG. Co-localization of BV2 cells and TNF-α, the immunofluorescence images were merged (orange). **B**) 6 h, 12 h and 24 h after stimulation by 1OD 1668ODN, the TNF-α expression was revealed by real-time PCR. **C**) Effects of ODN1668-induced phosphorylation of signaling molecule. BV2 cells were treated with ODN1668 for 5 min, 10 min, 20 min, 40 min, and medium as control. The phosphorylation level of AKT, ERK and JNK was analyzed using Western blotting. **D**) Relative density of p-AKT, AKT, p-ERK, ERK, p-JNK and JNK was analyzed using Photoshop, the ratio of phosphorylation and non-phosphorylation was shown in a line chart.

(n = 5, p < 0.05). The HE staining (Fig. 4A) and score of severity (Fig. 4B) of two groups showed that the severity of meningitis was significantly alleviated in the DEX group. Next, representative immunohistochemical staining results demonstrated that the level of TNF-α expression was also obviously inhibited by DEX (Fig. 4C).

To further confirm the decrease in TNF-α in DEX-pretreated mice, real-time PCR and immunoblotting were used for quantitative analysis. The real-time PCR and immunoblotting results both demonstrated that the level of TNF-α was decreased significantly in the brains of DEX-pretreated and ODN1668-treated mice (Fig. 4D-F).

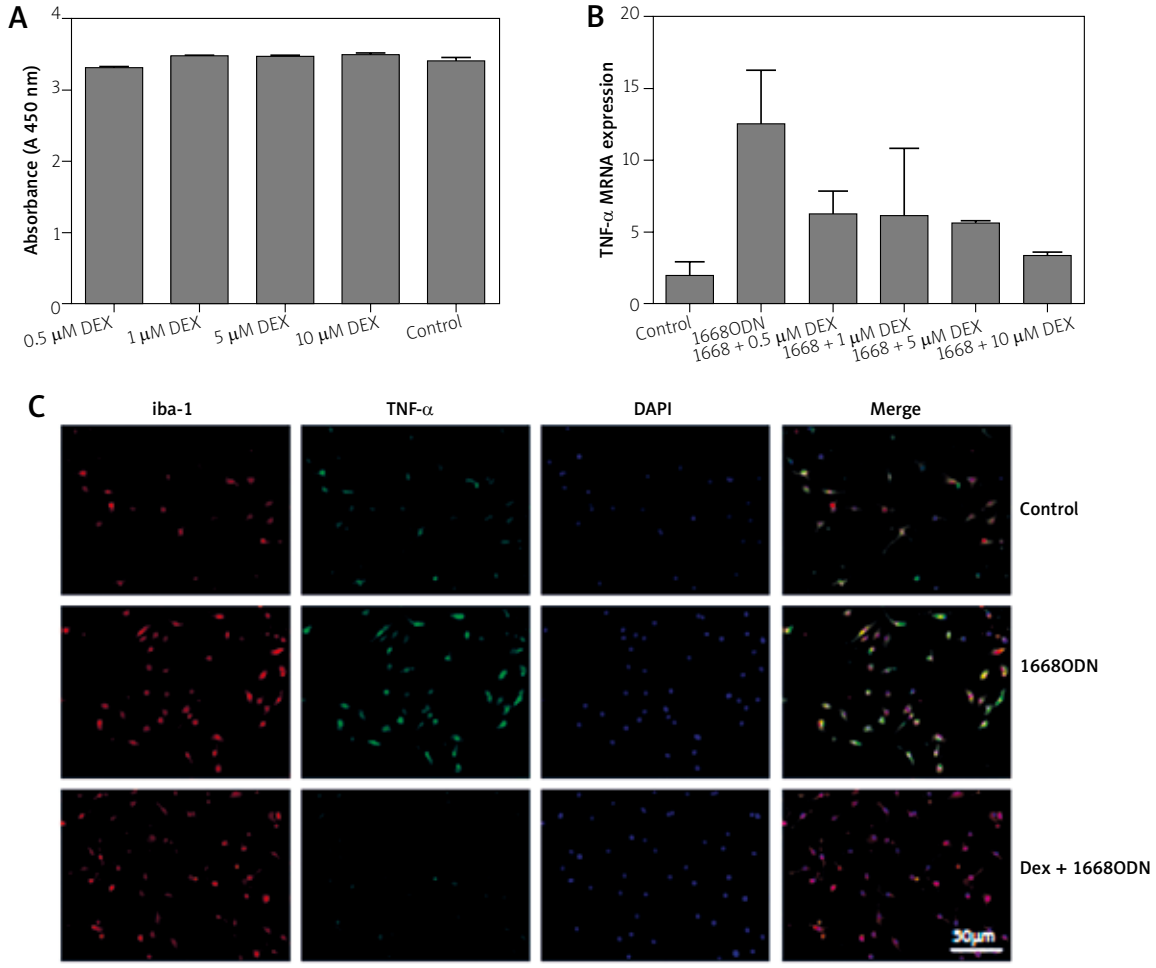

Fig. 3. Dexmedetomidine could inhibit microglia to express TNF-α. **A**) The CCK8 assay was used to evaluate the cell viability in different concentrations of DEX (0, 0.5 μM, 1.5 μM, 10 μM) and control. **B**) The BV2 were pre-treated with different concentrations of DEX (0.5 μM, 1 μM, 5 μM, 10 μM) for 15 min, then stimulated with 1OD ODN1668 for 6 h, real-time PCR assay was used to detect the expression of TNF-α. **C**) Primary mice microglia was stimulated first by DEX for 15 min then with 1668 ODN for 12 h, immunofluorescence photographs demonstrated that DEX could attenuate TNF-α expression compared with that stimulated with ODN1668 alone.

Discussion

Our study demonstrated that DEX exerted anti-inflammation effects concomitant with reduced TNF-α expression in microglia stimulated by unmethylated CpG via down-regulation of phosphorylated-AKT level. ODN 1668 could activate microglia to express TNF-α, and the effect lasts for 24h. Different results obtained may imply that differential concentrations of ODN1668 are applied in researches. In our study, a high dose of ODN1668 contributed to the activation of microglia in a shorter time. It has been reported that PI3/AKT is required for LPS-induced microglia activation [42]. We also demonstrated both microglia activation by CpG DNA and DEX inhibition via PI3/AKT pathway.

Unmethylated CpG-DNA motifs, conserved microbial patterns, are recognized by pattern recognition receptors. TLR-9 is essential for activation of innate immune cells by CpG DNA. TLRS gene transcript levels are up-regulated in the central nervous system in the neuroinflammation [34]. Activated microglia by CpG DNA can secret TNF-α and iNOS, which could induce neurons apoptosis [25]. Compared with LPS stimulation, the IL-12 induced by CpG DNA in the CNS is a decisive cytokine in pathophysiological processes in which T cells are involved. Therefore,

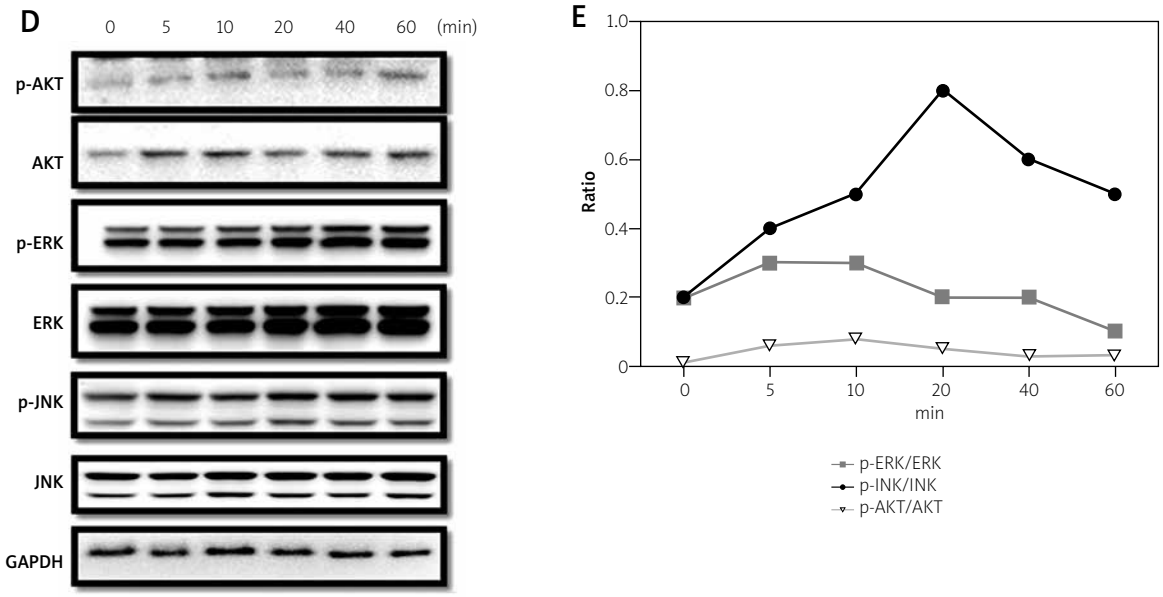

Fig. 3. Cont. Dexmedetomidine could inhibit microglia to express TNF-α. **D**) Inhibition effects of DEX on ODN1668-induced phosphorylation. The representative image of western blot analysis for p-AKT, AKT, p-ERK, ERK, p-JNK and JNK that was stimulated by DEX and ODN1668. GAPDH was used as the loading control. **E**) Relative density of p-AKT, AKT, p-ERK, ERK, p-JNK and JNK was analyzed using Photoshop, the ratio of phosphorylation and non-phosphorylation was shown in a line chart.

CpG DNA-activated microglia not only enhance T cell responses, but also induce effector molecules such as NO. However, microglia exhibit neuroprotection effects which are caused by CpG DNA in Alzheimer's disease [13]. The authors confirm that microglia express TLR9 at a high level, whereas astrocytes and neurons express it at a low level. In different diseases, activated microglia by CpG DNA play different roles [13]. In other organs and tissues, more researches about CpG DNA induced inflammation have been reported, such as lower respiratory tract inflammation [44]. Excessive TNF-α could cause damages in CNS, however, it is still evidenced that TNF-α plays a beneficial role in CNS under physiological conditions. TNF-α is initially to be constitutively expressed at low levels in the normal adult brains [5,6,51], and plays a critical role in homeostatic synaptic scaling [47]. TNF-α has multiple effects in the CNS ranging from glia activation and death to neuron survival and apoptosis.

DEX is widely used in the field of anesthesia, which could cross the blood-brain barrier and play an important role in the CNS function [2,22]. Studies have shown that DEX regulates inflammatory responses, plays anti-apoptotic roles and exerts neuroprotective effects in CNS [15,16]. Our study demonstrated the anti-inflammation effect of DEX in unmethylated CpG induced microglia activation. With the increase in concentration of DEX, the inhibition turned more obvious. There is a report that pretreatment with DEX could decrease TLR-4 expression to disclose the protective mechanism of DEX [20]. Our result implies that DEX may also decrease the TLR-9 expression. Microglia are activated by CpG DNA via TLR-9 and phosphatidylinositol 3-kinase-Akt pathway to express proinflammatory cytokines [45], DEX could inhibit the AKT-phosphorylation to block inflammation. The result is in accordance with other researches indicating that DEX is a potent suppressor of CNS inflammation [35].

The serine/threonine kinase Akt is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. It plays an important role in cell survival, growth, proliferation, angiogenesis, metabolism, and migration [31]; *i.c.v.* injection of ODN1668 time-reliant significantly increased AKT phosphorylation and after stimulation the levels of phosphorylation gradually increased. Pretreatment with DEX could inhibit TNF-α expression. Our data implied that DEX may be a potential medicine with

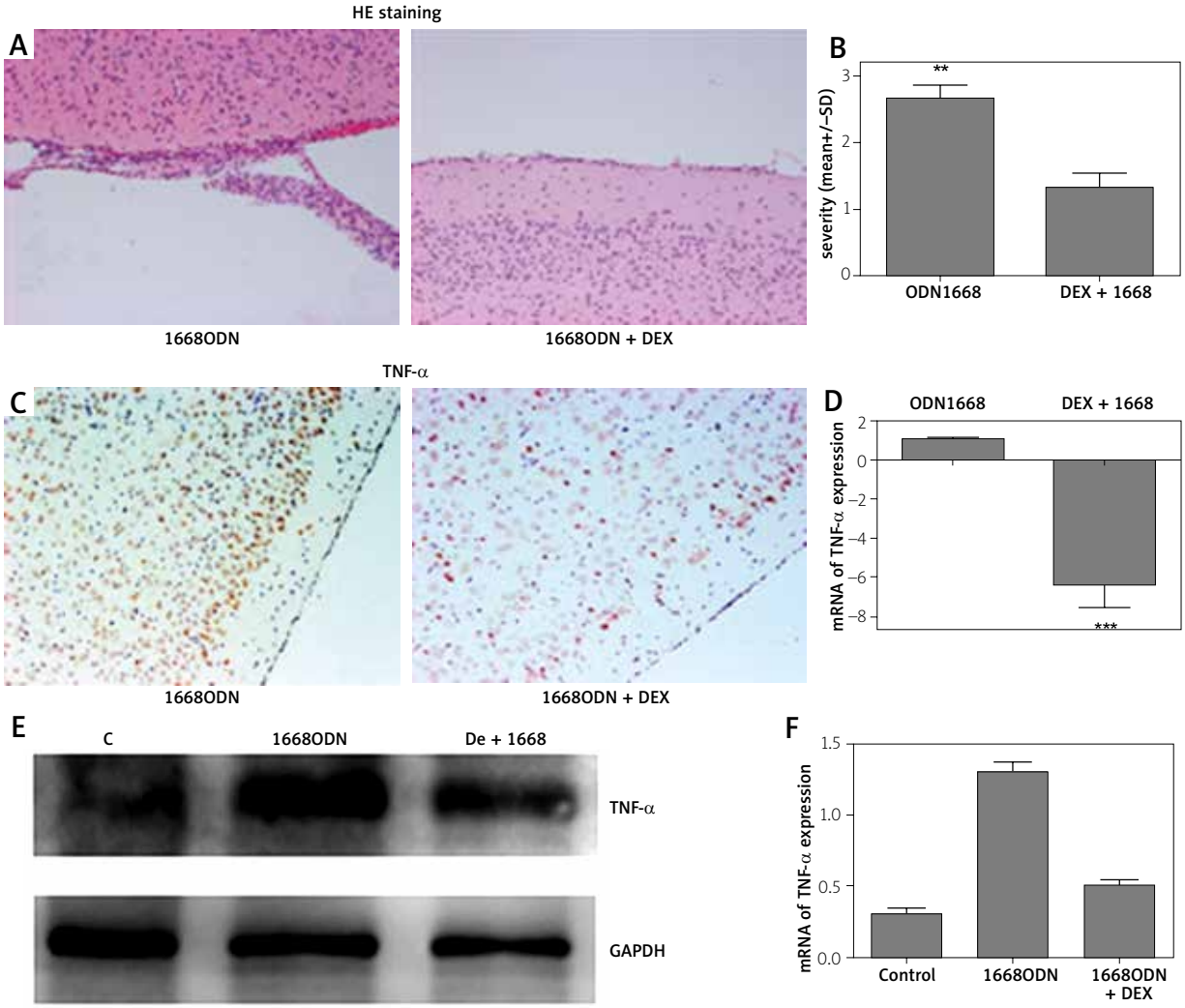

Fig. 4. Dexmedetomidine could inhibit 1668ODN-induced microglia TNA-α expression *in vivo*. **A**) C57BL/6 mice were pre-treated with DEX (50 µg/ml, *i.c.v.*) for 30 min, then 1OD ODN1668 was injected intracisternally for 3 d, representative histopathology photo was shown, ODN1668 injection alone was as control (n = 5 per group, magnification ×200). **B**) Score of meningitis severity in C57BL/6 mice treated with ODN1668 with or without DEX. $^{**}p < 0.01$ compared to DEX-pretreated group. **C**) Immunohistochemistry analysis of TNF-α in the brains that were given an intracisternal injection of ODN1668 with or without DEX (n = 5 per group, magnification ×200). **D**) 3 d after an intracisternal injection of ODN1668 with or without pretreated with DEX for 30 min, brain tissues were obtained and analyzed by real-time PCR. $^{***}p < 0.01$ compared to DEX-pretreated group. **E**) Mice received DEX (50 µg/ml *i.c.v.*) 30 min prior to ODN1668 treatment or not. All mice were sacrificed after 3 d, the protein was obtained from the brains, the western blot analyzed the TNF-α expression. Quantitative analysis results are shown in **F**.

anti-inflammatory effects in CNS. In neurodegenerative diseases, MAPK pathway also plays a critical role. In Parkinson's disease (PD), JNK, ERK and p38 MAPK may contribute differentially to the dopaminergic neuronal degeneration [19]. In amyotrophic lateral sclerosis (ALS), a ligand of growth hormone secretagogue receptor (GHS-R) 1a, protects motor neurons against glutamate excitotoxicity via activating ERK1/2 and phosphatidylinositol 3-kinase (PI3K)–Akt signaling [30].

In conclusion, our results showed that clinically relevant concentration of DEX suppressed TNF-α

expression in unmethylated CpG-activated microglia and AKT and ERK pathway might play a significant role in inflammatory or anti-inflammatory effects mediated by unmethylated CpG or DEX, providing a clue of therapeutic effects of DEX in neuronal inflammatory.

Acknowledgements

This study was jointly supported by the National Natural Science Foundation of China (NO. 81270429).

Disclosure

Authors report no conflict of interest.

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Original paper

Folia Neuropathologica

Nanofiber mat spinal cord dressing-released glutamate impairs blood-spinal cord barrier

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Folia Neuropathol 2016; 54 (4): 392-404 DOI: 10.5114/fn.2016.64818

Abstract

An excessive glutamate level can result in excitotoxic damage and death of central nervous system (CNS) cells, and is involved in the pathogenesis of many CNS diseases. It may also be related to a failure of the blood-spinal cord barrier (BSCB). This study was aimed at examining the effects of extended administration of monosodium glutamate on the BSCB and spinal cord cells in adult male Wistar rats. The glutamate was delivered by subarachnoidal application of glutamate-carrying electrospun nanofiber mat dressing at the lumbar enlargement level. Half of the rats with the glutamate-loaded mat application were treated systemically with the histone deacetylase inhibitor valproic acid. A group of intact rats and a rat group with subarachnoidal application of an 'empty' (i.e., carrying no glutamate) nanofiber mat dressing served as controls. All the rats were euthanized three weeks later and lumbar fragments of their spinal cords were harvested for histological, immunohistochemical and ultrastructural studies. The samples from controls revealed normal parenchyma and BSCB morphology, whereas those from rats with the glutamate-loaded nanofiber mat dressing showed many intraparenchymal microhemorrhages of variable sizes. The capillaries in the vicinity of the glutamate-carrying dressing (in the meninges and white matter alike) were edematous and leaky, and their endothelial cells showed degenerative changes: extensive swelling, enhanced vacuolization and the presence of vascular intraluminal projections. However, endothelial tight junctions were generally well preserved. Some endothelial cells were dying by necrosis or apoptosis. The adjacent parenchyma showed astrogliosis with astrocytic hypertrophy and swelling of perivascular astrocytic feet. Neurons in the parenchyma revealed multiple symptoms of degeneration, including, inter alia, perikaryal, dendritic and axonal swelling, and destruction of organelles. All the damage symptoms were slightly less severe in the rats given valproic acid treatment, and were absent from both the intact rats and the rats with 'empty' nanofiber mat dressing. These results demonstrate that glutamate-loaded nanofiber mat dressing can locally create glutamate levels capable of damaging BSCB and that the resulting damage can be mitigated with concurrent systemic valproate treatment.

Key words: *astrocyte, blood-spinal cord barrier, CNS damage, degeneration, endothelium, excitotoxicity, glutamate, neuron, valproate, vessels.*

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Introduction

The blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB) are composite structures the role of which is selective separation of the cerebral and spinal parenchyma from the contents of the blood vessel; these barriers play both a protective and regulatory role for the respective central nervous system (CNS) parenchyma [3]. The blood-brain barrier and BSCB are prone to damage from insults of varying origin, including local inflammatory processes, ischemia, hypoxia, mechanical trauma, and more.

The two barriers slightly differ structurally and functionally, mostly in ultrastructure of their endothelial cells [8,12,32,34,40]. These differences may be responsible for diverse susceptibility of BBB and BSCB to some pathological conditions [3]. Many authors suggest that disruption of BSCB or alteration of its permeability may be of key importance for the consequences of acute injury of the spinal cord [47] and for the development of some CNS pathologies, such as multiple sclerosis [37], amyotrophic lateral sclerosis [15,29], spinal cord ischemia [16,18,27], and neuropathic pain [4].

The main components of BSCB are endothelial cells, basement membrane and pericytes; some role is also played by perivascular astrocytes, the processes of which envelop small blood vessels [3,9]. An important role of astrocytes in the CNS is to support neurons under both normal physiological and pathological conditions, both by metabolic and regulatory means [10,25,44,46]. Due to the presence of active uptake and/or metabolic systems for some monoamine and amino acid neurotransmitters they can also contribute to the regulation of their interstitial levels [19,20,39]. After various CNS insults, astroglial cells invade the damaged region, become activated and hypertrophic, and, *inter alia*, start to express and release multiple trophic factors, including chemokines and cytokines [13].

An excessive glutamate level can cause damage and death of CNS cells and is also known as an important contributing factor in many CNS disorders [21,33]. Glutamate-damaged neurons show definite morphological alterations. Excitotoxic neuronal injury may correlate with BBB damage [7].

There is an ongoing search for new approaches to delivering various drugs into the CNS, and in particular those capable of chronic delivery. Our earlier data have shown the usefulness of subarachnoidal electrospun nanofiber mat dressing as the drug delivery system capable of long-term release of glutamate levels sufficient for damaging spinal cord motoneurons in the rat [36]. The aim of the present investigation was to examine the effects of this treatment on morphology of the various components of the BSCB, especially on endothelial cells.

Material and methods

Nanofiber mats

Drug-free ('empty') electrospun nanofiber mats as well as monosodium glutamate (MSG)-loaded electrospun nanofiber mats were prepared as described earlier [36].

Animals and experimental design

Adult male Wistar rats (starting body weight 250-300 g, n = 12) from the animal facility of the Mossakowski Medical Research Centre were used for the study. The rats were kept in three per opaque plastic cage (55 × 33 cm floor size) in an air-conditioned (60-70% relative humidity, 21 ± 2°C) room at a 12 h light/12 h dark day cycle (lights on at 7 a.m.), and were given free access to sterilized standard laboratory rat maintenance chow (Ssniff, Soest, Germany) and tap water.

The rats were randomized between four experimental groups of three rats each: 1) intact controls (group I), 2) rats with drug-free nanofiber mat application into the spinal cord subarachnoid space at the lumbar enlargement level (group II), 3) rats with MSG-loaded nanofiber mat (14.2 mg MSG/mg of the mat) into the subarachnoid space (group III); and 4) rats subjected to a surgical placement of the same glutamate-loaded mat into the subarachnoid space, which were additionally given by gavage one dose of the histone deacetylase inhibitor sodium valproate (Convulex syrup, 50 mg/ml, Gerot Pharmazeutika, Wien, Austria) daily, beginning on the surgery day (group IV). The initial valproate dose was 33.3 mg/kg body weight; the dosage was elevated by 8.3 mg/kg each day for 4 days and then was kept at 67 mg/kg body weight for the remainder of the study period.

Subarachnoidal implantation of nanofiber mat pieces (5 × 5 mm size) into rat spinal cords (at the lumbar enlargement level) was performed as described earlier [1]. Three weeks after the surgery, all rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and decapitated. The L1-L6 segments

of their spinal cords were harvested and immediately fixed in 4% formaldehyde solution in PBS and next embedded in paraffin by a standard procedure. The paraffin blocks were then cut and processed for histological and immunohistochemical methods.

All animal use procedures were in agreement with the European Communities Council Directive on the protection of laboratory animals (86/609/EEC) and with the current Polish law. All efforts were made to reduce animal discomfort and the number of rats used. The protocol of the study has been accepted by the 4th Local Animal Experimentation Ethics Committee at the National Medicines Institute, Warsaw, Poland (Certificate No. 43/210).

Histology and immunohistochemistry

The paraffin-embedded spinal cord samples were cut crosswise into 8 µm sections. After deparaffinization and rehydration in a water-ethanol solution series by standard procedures, some of the sections were stained with hematoxylin-eosin mixture solution using a routine procedure. The sections selected for immunohistochemistry were first incubated with rabbit polyclonal anti-GFAP antibody (Dako cat. no. Z0334; diluted 1 : 4000). Next, the sections were incubated with goat anti-rabbit antibody (Beckman Coulter Inc., France, cat. no. IM0830; dil. 1 : 100) and then with streptavidin-horseradish peroxidase solution (Beckman Coulter cat. no. IM0309; dil. 1 : 500). The resulting immune complexes were visualized by a routine procedure with diaminobenzidine as the chromogen and then counterstained with hematoxylin. The intensity of GFAP immunostaining was assessed by an experimenter blinded to the samples identity, using a light microscope (Nikon, Japan) equipped with a CCD camera and a PC-based image analyzer system. Specificity of the staining was tested by running the same procedure on the respective sister sections with the primary antibody absent from the incubation mixture; the control sections revealed no immunosignal.

Transmission electron microscopy

Samples of lumbar spinal cord meant for electron microscopy were instantly fixed in a formaldehyde-glutaraldehyde (2%/2.5%) solution in cacodylate buffer pH 7.4 for 4 h. Next, they were cut into smaller fragments, rinsed in the cacodylate buffer, post-fixed in 1.0% OsO_4 solution for 1 h, dehydrated by a standard procedure, embedded in epon resin, and cut into 40 µm-thick sections. The sections were stained with 1% toluidine blue and examined in a light microscope for block selection. The selected blocks were then cut into ultrathin sections that were stained with uranyl acetate and lead citrate and then examined with a model JEOL 1200EX (Jeol, Japan) electron microscope by an experimenter blinded to the samples identity.

Results

Light microscopy morphological and immunohistochemistry studies

Spinal cords from the intact rats and rats carrying 'empty' nanofiber mat dressing showed normal parenchyma and BSCB morphology. Spinal cords from the rats carrying MSG-loaded spinal cord dressing (group III) revealed the presence of multiple intraparenchymal microhemorrhages of varying sizes (Figs. 1 and 2). The capillaries in the vicinity of the dressing (both in the meninges and the white matter) were leaky and showed considerable swelling; there was no difference in these characteristics between rats with and without systemic valproic acid treatment (group IV and group III, respectively).

Immunohistochemistry revealed astrogliosis, presence of numerous activated hypertrophic astrocytes (Fig. 3) and swelling of the astroglial end-feet processes located close to the leaky vessels both in group III and group IV; however, the symptoms were more severe in group III. No signs of astroglial reaction were found in the control group with 'empty' subarachnoidal nanofiber mat dressing (Fig. 3).

Electron microscopy studies

In the intact controls (group I) and in the rats with 'empty' nanofiber mat dressing (group II) (Fig. 4), electron microscopy showed proper morphology of blood vessels, neurons and glial cells. The structure of microvascular basement membrane was homogenous and tight (Figs. 2-4); the vicinity of the vessels showed the presence of normal-looking astrocytic end-feet and scanty intercellular space.

In group III rats, neurons revealed both cytoplasmic and mitochondrial edema, organelle decay, endoplasmic reticulum atrophy and cytoplasmic microvacuolization. These alterations were more extensive in postsynaptic dendrites (Fig. 5A) than in neuronal perikarya (Fig. 5B). Similar changes were seen in the cyto-

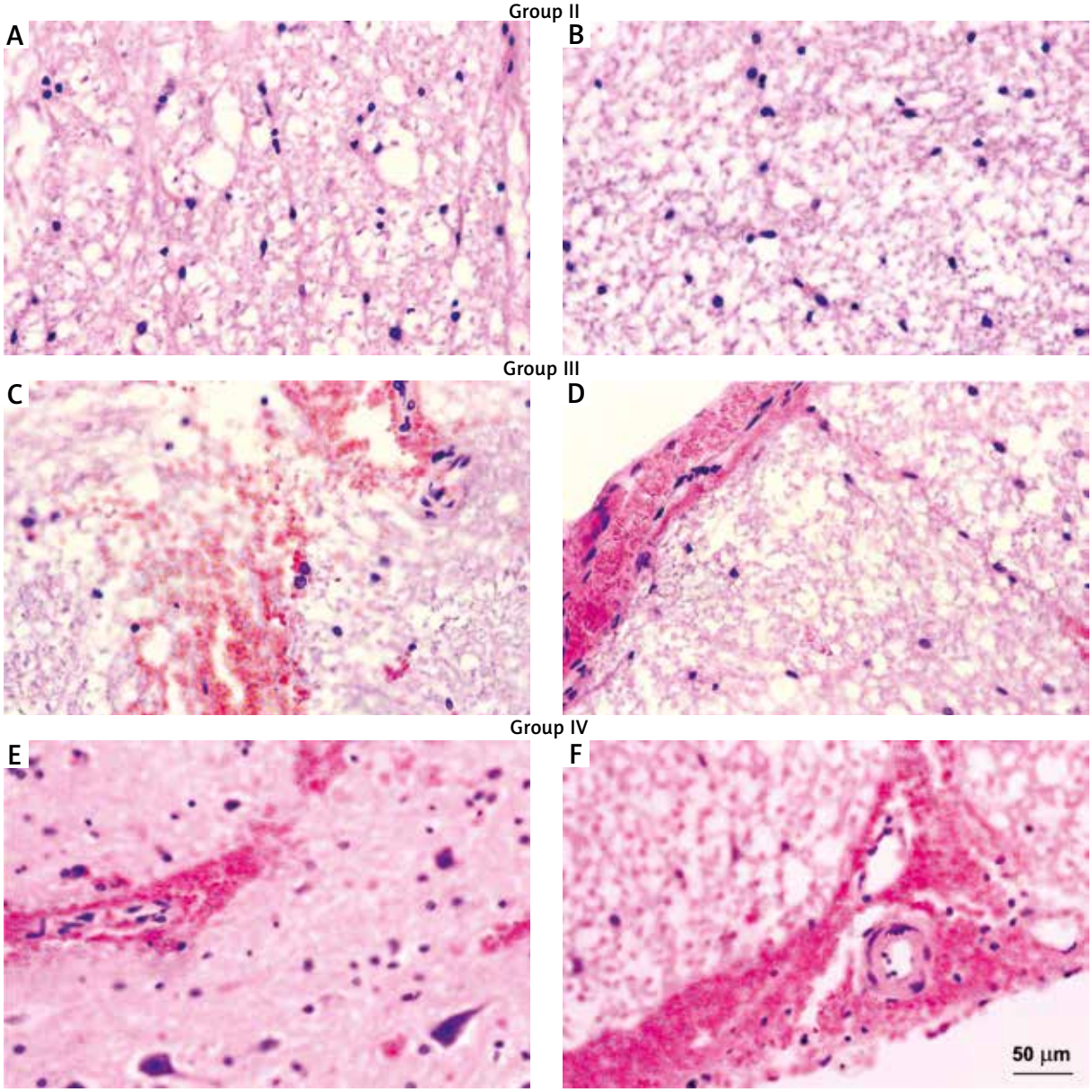

Fig. 1. Microhemorrhages in the vicinity of the nanofiber mat dressing (hematoxylin-eosin staining). The spinal cord section from a group II rat (control with 'empty' mat dressing) shows no visible microhemorrhages in the parenchyma. The sections from a group III rat and a group IV rat show the presence of microhemorrhages both within the parenchyma (left column) and in the subarachnoid space (right column).

plasm of both astroglial cells (Fig. 5C), small vessel endothelial cells and pericytes (Fig. 5D). The vessels showed endothelial cells damage of varying intensity and thickened basement membrane; edema of perivascular end-feet processes and neuropil, and expansion of the interstitial space were apparent as well. Some vessels showed endothelial cells necrosis with destruction of cell membrane and deterioration of tight junctions (Figs. 5B and 6A). A typical pattern of apoptotic changes of the endothelium was but rarely seen (Fig. 6B). More frequently, the endothelium showed a mixed pattern of overlapping apoptotic and necrotic changes (Fig. 6C-D), with increased cytoplasm density and relatively well-preserved organelles in some (shrunken) cells, and cytoplasmic edema and vacuolization, organelle and cell membrane decay in the other cells. Most of the damaged endothelial cells had an irregular luminal surface, with multiple

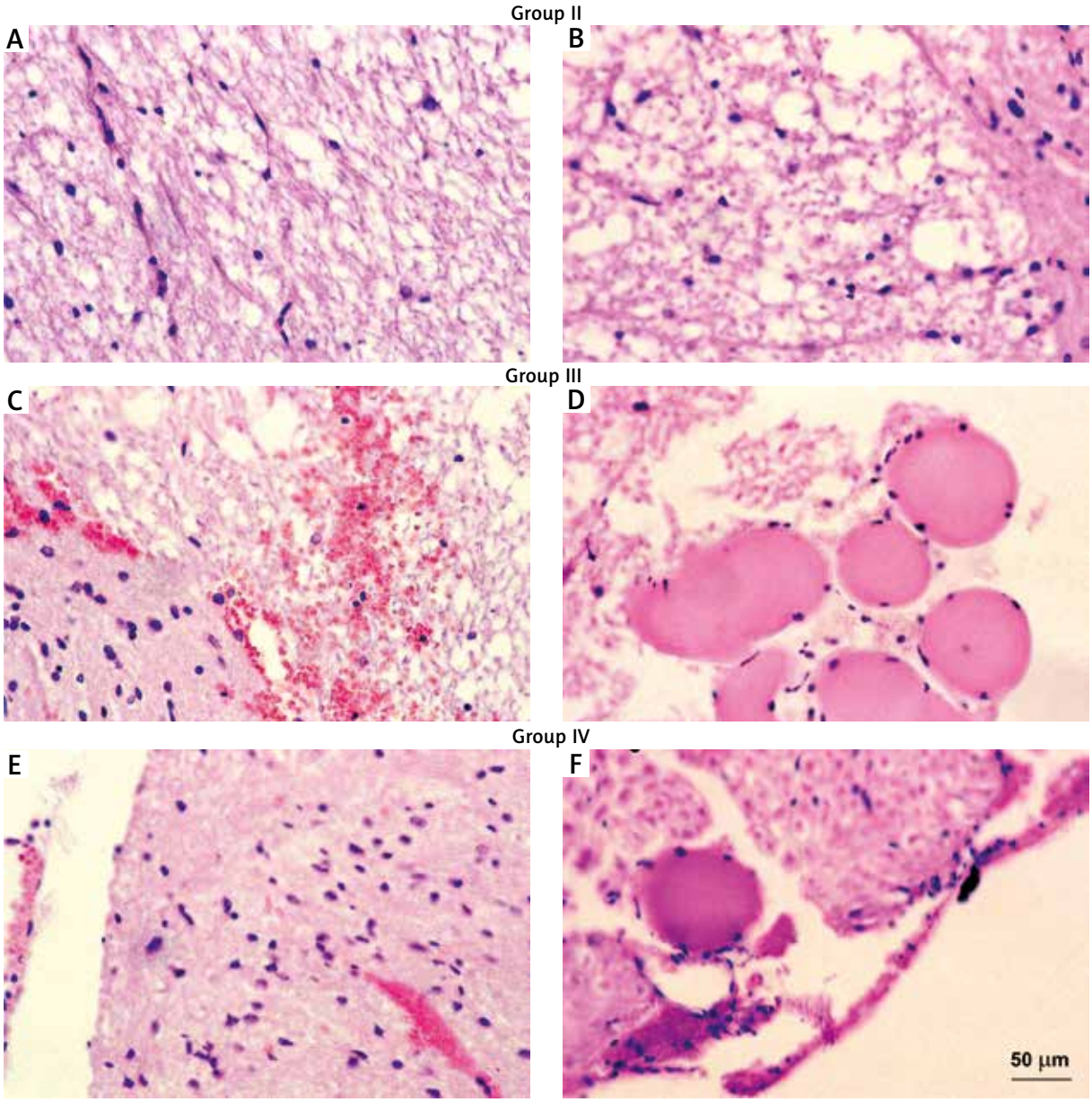

Fig. 2. Microhemorrhages (left column) and dilation of spinal cord capillary vessels (right column) in the vicinity of the nanofiber mat dressing (hematoxylin-eosin staining). The section from a group II (control) rat shows no vessel dilation. The sections from group III and group IV rats show the presence of dilated capillaries/microvessels in the parenchyma, meninges and posterior funicles.

intraluminal cytoplasmic microvilli-like protrusions, increased numbers of plasmalemmal vesicles, cytoplasmic vacuoles and irregular endoplasmic reticulum channels (Fig. 6C-E). Most endothelial tight junctions remained closed (Fig. 6E); opened tight junctions were occasionally seen, especially in the proximity of the basement membrane. The basement membrane of the vessels was mostly thickened and stratified, with embedded fragments of pericytes (Fig. 6E). The adjacent parenchyma showed considerable edema and decay of neuropil and astrocytic perivascular processes (Fig. 6C-D).

In the spinal cords of group IV rats, the edema of the endothelium and perivascular astrocytic end-feet was of clearly lesser severity (Figs. 7A-B); the endothelial cells showed nearly normal luminal cellular membrane (with only occasional intraluminal protrusions), mostly well-preserved mitochondria, ribo-

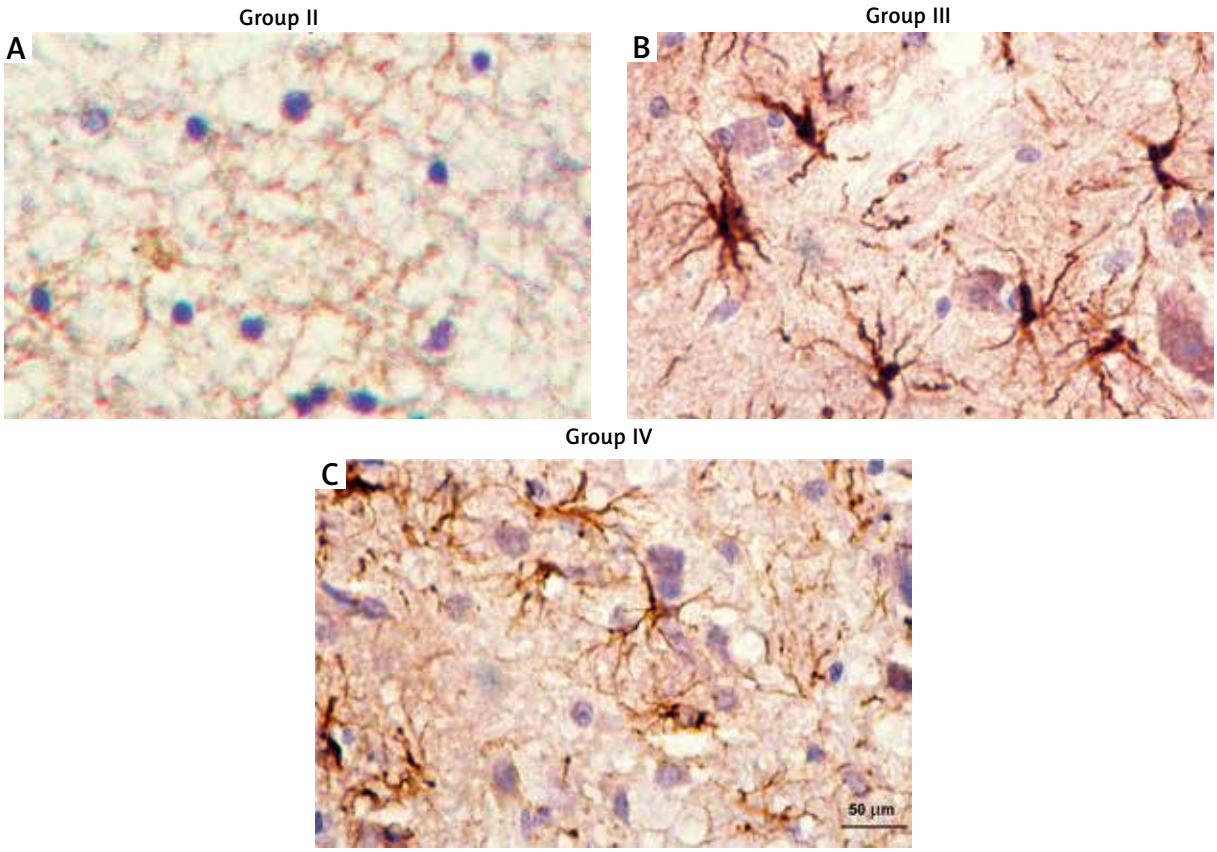

Fig. 3. Perivascular astrocytes of spinal cord capillary vessels in the vicinity of the nanofiber mat dressing (GFAP immunostaining). The spinal cord section from a group II rat shows astroglia with normal morphology. The spinal cord section from a group III rat shows activation (hypertrophy of perikarya and processes) in a majority of astroglial cells, and clasmatodendrosis (signs of degeneration) in some astrocytes. The spinal cord section from a group IV rat shows astroglial activation of lesser severity and no visible clasmatodendrosis.

somes and endoplasmic reticulum; single autophagic vacuoles were but sporadically seen. In summary, all the MSG-loaded electrospun nanofiber mat dressing-induced alterations were more severe in group III than in group IV rats.

Discussion

Glutamate belongs to the most extensively studied neurotransmitters. Its physiological content in the nervous tissue corresponds with the metabolic demand of the cells, and the BBB and BSCB protect CNS parenchyma from an influx of excessive extra-CNS glutamate amounts [26]. It is common knowledge that CNS glutamate concentration is greatly elevated in many CNS pathologies [23,35,43,48]. It has been demonstrated as well that the considerable ischemia-related elevation of intracerebral glutamate results in BBB damage and brain edema [41]. Glutamate is also assigned an important role in the pathomechanisms of many CNS pathologies, e.g., of epilepsy, ischemia and amyotrophic lateral sclerosis, and in acute brain or spinal cord injury [2,26,28,30].

The present study is a continuation of our earlier investigation that demonstrated a toxic effect of exogenous glutamate delivered by subarachnoidal application of MSG-loaded electrospun nanofiber mat dressing on spinal cord motoneurons and a neuroprotective action of concurrent sodium valproate treatment [36]. This study was aimed at determining the potential of subarachnoidal electrospun nanofiber mat dressing for delivering MSG in the amount sufficient for inducing BSCB damage, and at defining the effect of the exogenous glutamate on perivascular cells of the spinal cord parenchyma. We have demonstrated that the chronic action of the mat-released glutamate damages BSCB. Blood

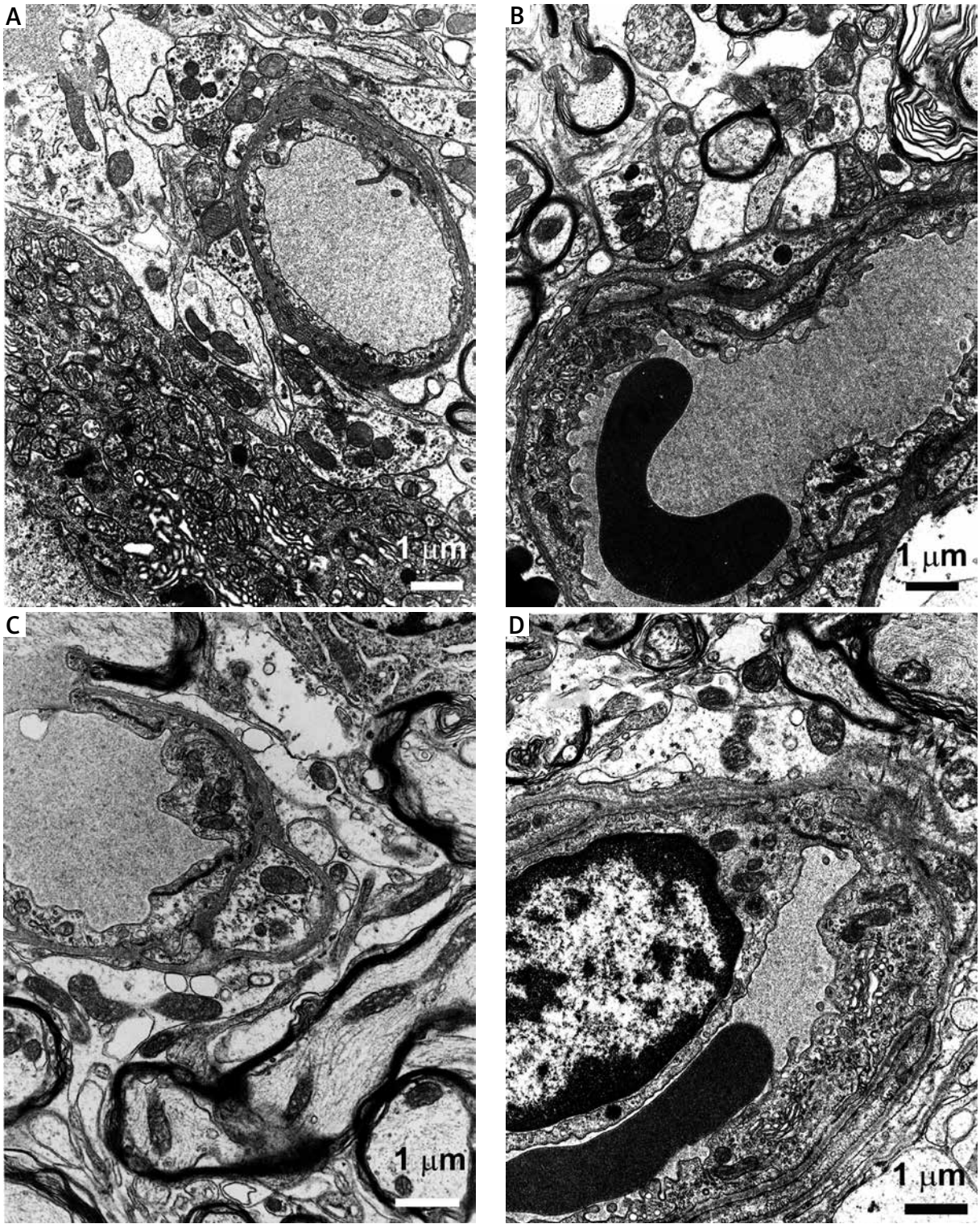

Fig. 4. Representative electron micrographs of spinal cord sections from a group II rat. **A)** Well-preserved organelles, e.g. mitochondria, are visible both in neuronal cytoplasm and in processes of neuronal, glial as well as endothelial cells of a normal-looking capillary. **B-C)** Microvessels with normal ultrastructure, smooth luminal surface of the endothelium and well-preserved endothelial tight junctions (upper left corner). The cytoplasm of endothelial cells and pericytes shows the presence of small dark mitochondria, normal-looking endoplasmic reticulum and few vacuoles. **D)** The endothelial nucleus has clear euchromatin and evenly distributed heterochromatin.

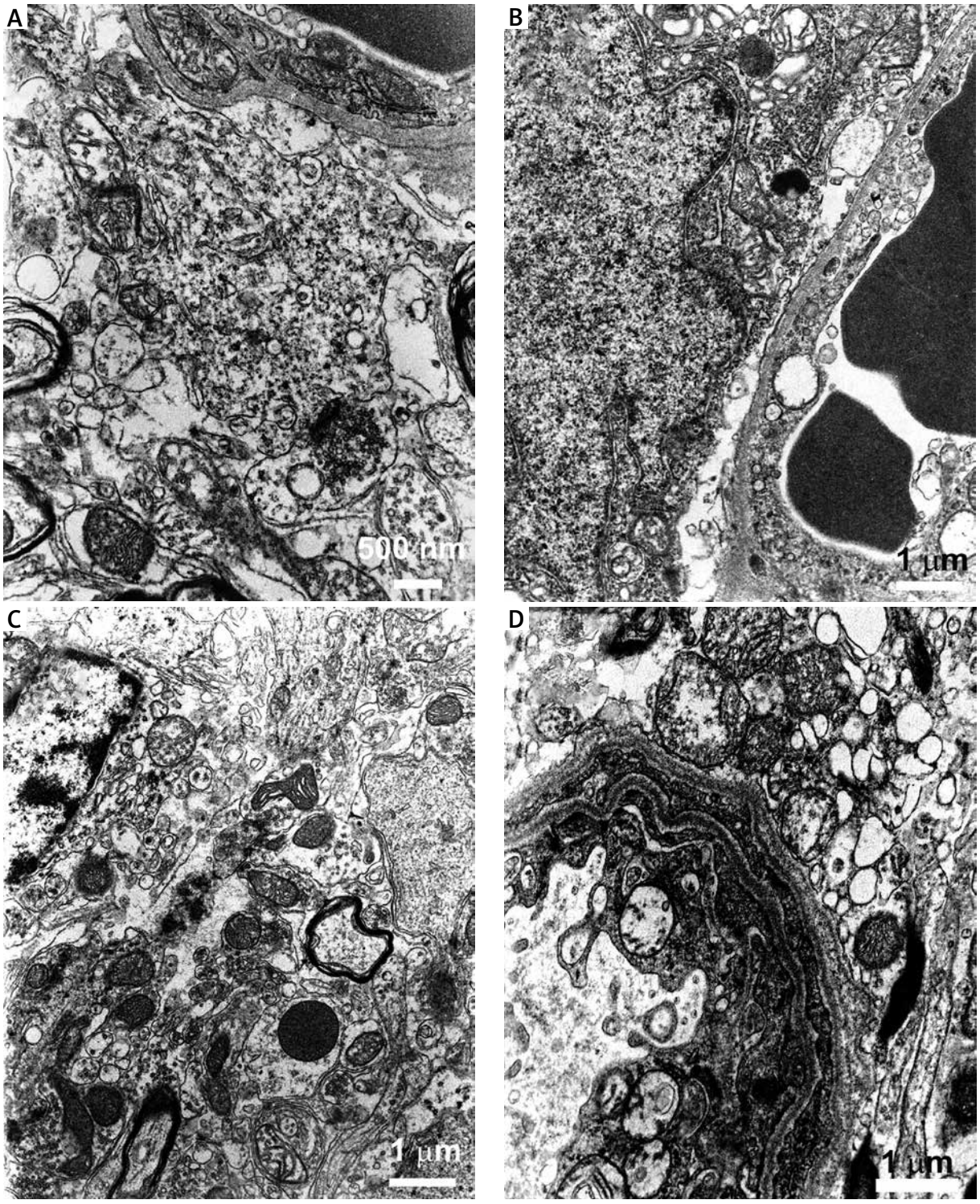

Fig. 5. Representative electron micrographs of spinal cord sections from a group III rat showing degenerative changes. **A)** Neuron showing decay of organelles, dendrite degeneration, and microvacuolization. Perivascular parenchyma is swollen. **B)** Neurons show perikaryal degeneration as well as edema and decay of organelles. In the lower right corner of the photograph, a fragment of a necrotic endothelial cell is visible, which shows destruction of cell membrane. **C)** Degeneration and decay of astrocytic organelles. **D)** Degenerative alterations in a perivascular astrocyte and capillary endothelium.

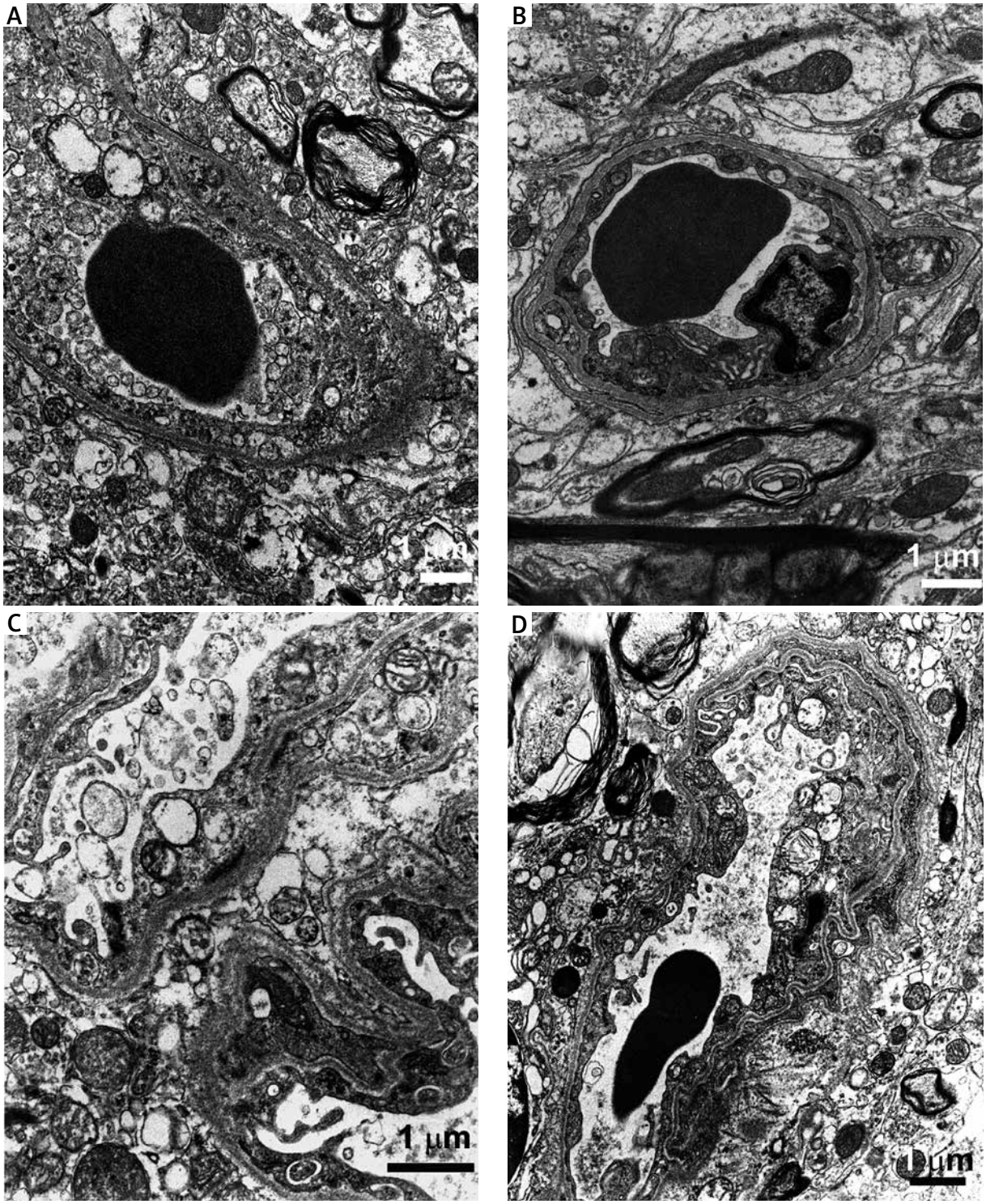

Fig. 6. Representative electron micrographs of spinal cord sections from a group III rat showing cell death. **A)** Damage of cell membrane in capillary endothelium. **B)** Early apoptotic changes in an endothelial cell. **C-D)** Coexistence of apoptotic and necrotic cells. Perivascular zone shows decay of the neuropil and astrocytic processes.

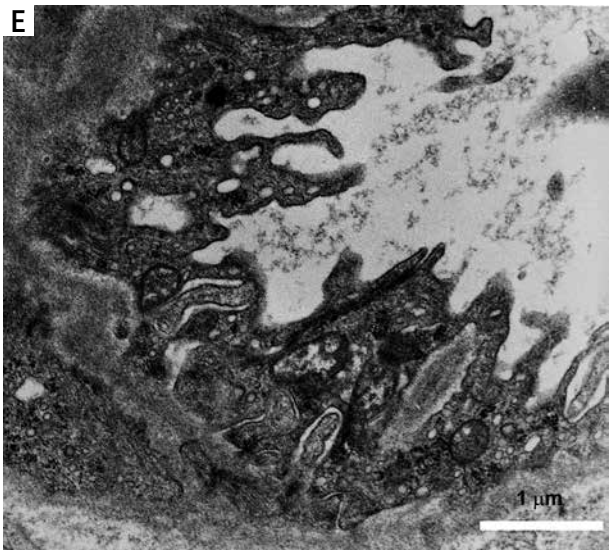

Fig. 6 cont. Representative electron micrographs of spinal cord sections from a group III rat showing cell death. **E)** Thickened basement membrane with visible fragment of a pericyte. Capillary endothelium shows the presence of multiple microvacuoles and carries intraluminal microvilli-like processes.

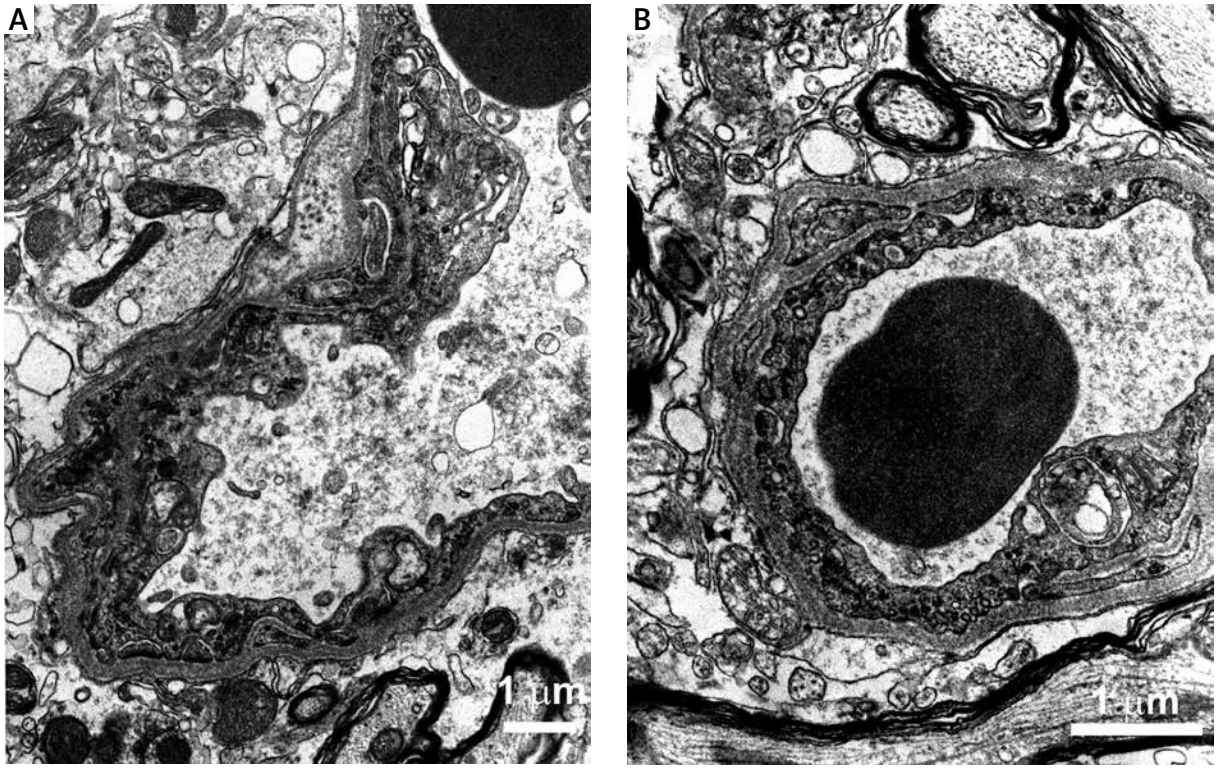

Fig. 7. Representative electron micrographs of spinal cord sections from a group IV rat. **A-B)** Moderate edema of vascular endothelium and perivascular astrocyte end-feet processes. Endothelial cells show almost smooth luminal cell membrane, mostly well-preserved organelles and considerably less cytoplasmic microvacuoles than in the group III (Fig. 6E) rat.

vessels become dilated and permeabilized, and their vicinity showed the presence of edema and multiple microhemorrhages. Our studies have also revealed a strong astrocytic reaction in the vicinity of the damaged blood vessels, which demonstrated itself in marked astrogliosis and cell activation. However, some of the astrocytes showed clasmatodendrosis evidencing cell degeneration. Electron microscopy has also demonstrated the presence of 'empty' astrocytic end-feet processes evidencing perivascular edema. Considerable alterations were also visible in the cytoplasm and organelles of endothelial cells and in the adjacent basement membrane. The vicinity of the vessels showed also the presence of degenerating neurons. Interestingly, the glutamate-evoked alterations of the BSCB did not significantly affect the state of these cells in that their condition, while poor, was not worse than that of the neurons more distal to the damaged vessels [36]. It is believed that the basis of the glutamate-induced cell damage are, *inter alia,* an enhanced generation of free radicals and excessive calcium ions levels [11,13,14,17,38,45], which ultimately cause permeabilization of blood vessel walls by damaging BBB/BSCB integrity via a variety of signal transduction pathways and/or by direct action on endothelial tight junctions [6].

Glutamate excitotoxicity causes severe disturbances in protein and lipid metabolism by activating AMPA, NMDA and kainate glutamate receptors [24,26], and in particular the endothelial NMDA receptor subset [5,22]. Excessive stimulation of the NMDA receptors causes imbalance of Na^+ ions across plasma membranes [31], increased influx of Ca^{2+} ions and activation of a wide spectrum of intracellular enzymes, including kinases, proteases and phospholipases [26], and promotes free radical-related oxidative stress in endothelial cells [42]. The latter in turn causes activation of NMDA receptors, resulting in forming of a metabolic vicious circle that intensifies functional and structural disturbances of cell membranes and increases BBB/BSCB permeability.

All said, the results of this study indicate that MSG-loaded nanofiber mat dressing can create glutamate concentrations capable of damaging both the BSCB and the neighboring parenchyma in the rat. Whereas the glutamate released from the dressing damages them both considerably, the concurrent systemic administration of the histone deacetylase inhibitor valproic acid alleviates the detrimental consequences of the long-term action of excessive glutamate levels on parenchyma more than those seen in the barrier.

Acknowledgments

This work was supported by grant no. NN 401 014640 from the Ministry of Science and Higher Education of Poland, and by statutory funds from the Mossakowski Medical Research Centre, Polish Academy of Sciences. The authors thank Dr. Tomasz Kowalczyk of the Institute of Fundamental Technological Research, Polish Academy of Sciences, for his consulting the preparation and *in vitro* characterization of the electrospun nanofiber mats.

Disclosure

Authors report no conflict of interest.

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Folia Neuropathologica

Bilateral striatal necrosis caused by *ADAR* mutations in two siblings with dystonia and freckles-like skin changes that should be differentiated from Leigh syndrome

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Folia Neuropathol 2016; 54 (4): 405-409 DOI: 10.5114/fn.2016.64819

Abstract

Pathogenic molecular variants in the ADAR gene are a known cause of rare diseases, autosomal recessive Aicardi-Goutières syndrome type 6, severe infantile encephalopathy with intracranial calcifications and dominant dyschromatosis symmetrica hereditaria, demonstrated mainly in Asian adults. Recently, they have been also found in patients with nonsyndromic bilateral striatal necrosis accompanied by skin changes of the freckles-like type.

Here, we present Polish siblings with acute onset and slowly progressive extrapyramidal syndrome with preserved intellectual abilities and basal ganglia changes found in MRI. A Leigh syndrome was considered for a long time as the most frequent cause of such lesions in children. Finally, two molecular variants in non-mitochondria-related ADAR gene c.3202+1G>A (p.?) and c.577C>G (p.Pro193Ala) were revealed by whole exome sequencing.

We suggest that bilateral striatal necrosis should be always differentiated from LS to prevent the diagnosis delay. The striatal involvement accompanied by the presence of freckles-like skin changes should direct differential diagnosis to the ADAR gene mutations screening.

Key words: *bilateral striatal necrosis, ADAR gene, whole exome sequencing, LS differentiation.*

Introduction

Pathogenic molecular variants in the *ADAR* gene [OMIM*146920] are a known cause of an Aicardi-Goutières syndrome type 6 (ASG6) [OMIM#615010], an autosomal recessive severe infantile encephalopathy with intracranial calcifications [10] and a dyschromatosis symmetrica hereditaria (DSH) [OMIM #127400], a rare dominant disease demonstrated mainly in Asian adults [8]. Recently, they have been also found in patients with nonsyndromic bilateral striatal necrosis (BSN) [6,7] that is a frequent but

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nonspecific MRI feature observed in patients with an extrapyramidal syndrome, some of which had DSH [1]. Bilateral striatal necrosis was the most frequently reported in individuals with mitochondrial pathology presenting Leigh syndrome (LS) and *MTATP6* mutation [3], thiamine metabolism dysfunction syndrome related to *SLC25A19* pathogenic variants [11], and glutaric aciduria I with *GCDH* variants [4], but rarely in Wilson's and Huntington diseases and other of inherited etiology as well as of certain acquired causes [1]. The prognosis for BSN is variable, with patients completely recovered and others developing severe dystonia or a more akinetic-rigid phenotype [7]. The *ADAR* gene (1q21.3) codes for specific deaminase which converses adenosine to inosine in double-stranded RNA, influences glutamate receptor transcripts and acts as a suppressor of type I interferon signaling [10]. It is ubiquitously expressed in all tissues, but until today it has not been known why the signs of its dysfunction are limited to the nervous system and skin.

Case study

Here, we present two Polish siblings, the only children of unrelated parents, born after uneventful pregnancy and delivery. They both demonstrated acute onset with an episode of ataxia that occurred after a nonspecific infantile febrile infection followed by slowly progressive extrapyramidal syndrome (Table I). In infancy their development was mildly delayed, mainly in motor skills as they moved on their fours up to 2 and 3 years (Case 1 and 2, respectively). When they started to walk independently, their movements were disturbed by an increased muscle tone with worsening during emotional stress. Tendency to retropulsion of the head, involuntary movements of facial muscles, athetotic movements of digits as well as dysarthric speech, dysphagia and frequent choking during eating were noticed. They both had small mild freckles-like skin changes on their faces and dorsal surfaces of hands. Both children were small for their age (weight and length < 2.5 SD, OCF < 3SD). Magnetic resonance imaging (MRI) examination (Fig. 1A-C) showed bilateral lesions in putamen on T2-weighted and FLAIR sequences that were suggestive for LS. Magnetic resonance spectroscopy (MRS) did not show any abnormalities. Muscle biopsy investigations did not reveal morphological changes and respiratory chain dysfunction.

During the following years, the clinical course of the disease was slowly progressive with the dominance of dystonic disorder, but intellectual ability was relatively preserved. At present (14 and 12 years), the siblings are wheel-chair bound and need full assistance with dressing and feeding.

Molecular screening for *SURF1*, *SCO2, POLG, MTATP6, MTTL1,* and *MTTK* mutations most frequently detected in Polish LS patients [9] was negative. Whole exome sequencing (WES) did not reveal deleterious mutations in genes responsible for known mitochondrial diseases (MD). Finally, two rare molecular variants (Fig. 1D, E) in non-mitochondria related *ADAR* gene (Ref Seq. NM_001111.4; NP_001102.2) included one novel splicing variant c.3202+1G>A (p.?) and a known recurrent substitution c.577C>G (p.Pro193Ala) were identified by thorough filtration of WES data and confirmed by Sanger sequencing in both siblings.

The study was approved by the Bioethical Commission of the CMHI.

Discussion

In the reported family the extended metabolic and mitochondrial investigations have been inconclusive for approximately 14 years. Our patients developed signs of the disease as a sequel of the infection, the finding pointed also by Livingston *et al*. [7]. Freckles-like skin changes on the face and the dorsal surface of hands were noticed in their medical documentation but were neglected in the differential diagnostics. Bilateral putaminal lesions found in MRI were not specific enough to establish a final diagnosis but together with the secondary abnormalities in lactate and alanine concentrations led us to consider for a long time a mitochondrial disease as the most frequent cause of such features in children. Our patients similar to those described in a cohort of children with nonsyndromic BSN [7] did not have any signs of calcification in the striatum or other localizations that were reported by Kumar *et al*. [5] in AGS brains.

It is worth noting that the c.577C>G (p.Pro193Ala) substitution was detected earlier in at least eleven AGS6 families [7,10] and it was identified in the general population. The minor allele frequency (MAF) already recorded in ExAC database of 65000 exomes was 0.002142 (http://exac.broadinstitute.org), in 1000 Genomes was 0.0013 (http://browser.1000genomes.org), and in our in-house-made 400 exomes database it was evaluated as 0.0014. It is located in

Table I. Clinical and biochemical characteristics of the siblings with *ADAR* mutations

Factor	Case 1	Case 2
Gender	Male	Female
Birth data (weight/length/OCF/Apgar points)	3050 g/53 cm/36 cm/9	3400 g/56 cm/33 cm/10
Motility:		
Sitting	6 mo	7 mo
Start to walk	10 mo	14 mo
Stop to walk independently	4 yrs	> 4 yrs
First symptoms	12 mo	8 mo
Extrapyramidal signs	3 yrs: dystonia, elevated tendon reflexes, striatal great toe	4 yrs: axial and limbs hypotonia, dystonic hypertonia, elevated and polyclonal reflexes
Neurologic examination:		
EEG	Normal	Normal
EMG	Normal	Normal
Fundoscopy	Normal	Normal
Nerve conduction	Sensory-motor neuropathy of axonal type	Sensory-motor neuropathy of axonal type
MRI	Symmetrical changes in putamen	Symmetrical changes in putamen
MRS	Normal	Normal
Leiter International Performance Scale*	100	112
CSF examination:		
Pleocytosis	1/ml	
Protein content (ref. 200-450 mg/dl)	239 mg/dl	
Glucose (ref. 45-80 mg/dl)	50 mg/dl	
Lactate (ref. < 2.2 mmol/l)	1.1 mmol/l	1.57
Alanine (ref. 65 µmol/l)	98.8 µmol/l	51.9
Threonine	67.4 µmol/l	
Laboratory tests for IEM:		
Plasma lactate (ref. < 2 mmol/l)	3.08, 2.42	3.01
Plasma alanine (ref. < 450 µmol/l)	706.8 µmol/l	
Threonine	170.9 µmol/l	
Urine biopterin concentration (based on creatinine (C) conc.)	4594 nmol/l (2.7 nmol B/µmol/C)	
Urine neopterin concentration (based on creatinine (C) conc.)	2027 nmol/l (1.2 nmol//µmol/C)	
Muscle biopsy investigations:		NA
Morphology	No changes	
OXPHOS function	Normal	
Amount of E1-alfa subunit of PDHC	79.5% of ref.	
Molecular screening for *SURF1*, *SCO2*, *MTATP6*, *POLG*, *MTTL1*, *MTTK* common mutations**	Negative	NA

*Leiter RG. Instruction Manual for the Leiter International Performance Scale. Stoelting Co., Wood Dale 1979
c.845_846delCT, c.312_321delinsAT (*SURF1***), c.418G>A (***SCO2***), m.8993T>G, m.8993T>C (***MTATP6***); c.1399G>A, c.2243G>C, c.2542G>A (***POLG***); m.3243A>G (***MTTL1***), m.8344A>G (***MTTK***)

mo – months, yrs – years, EEG – electroencephalography, EMG – electromyography, MRI – magnetic resonance imaging, MRS – magnetic resonance spectrometry, IEM – inborn errors of metabolism, OXPHOS – oxidative phosphorylation system, PDHC – pyruvate dehydrogenase complex, NA – not analyzed, ref. – control values

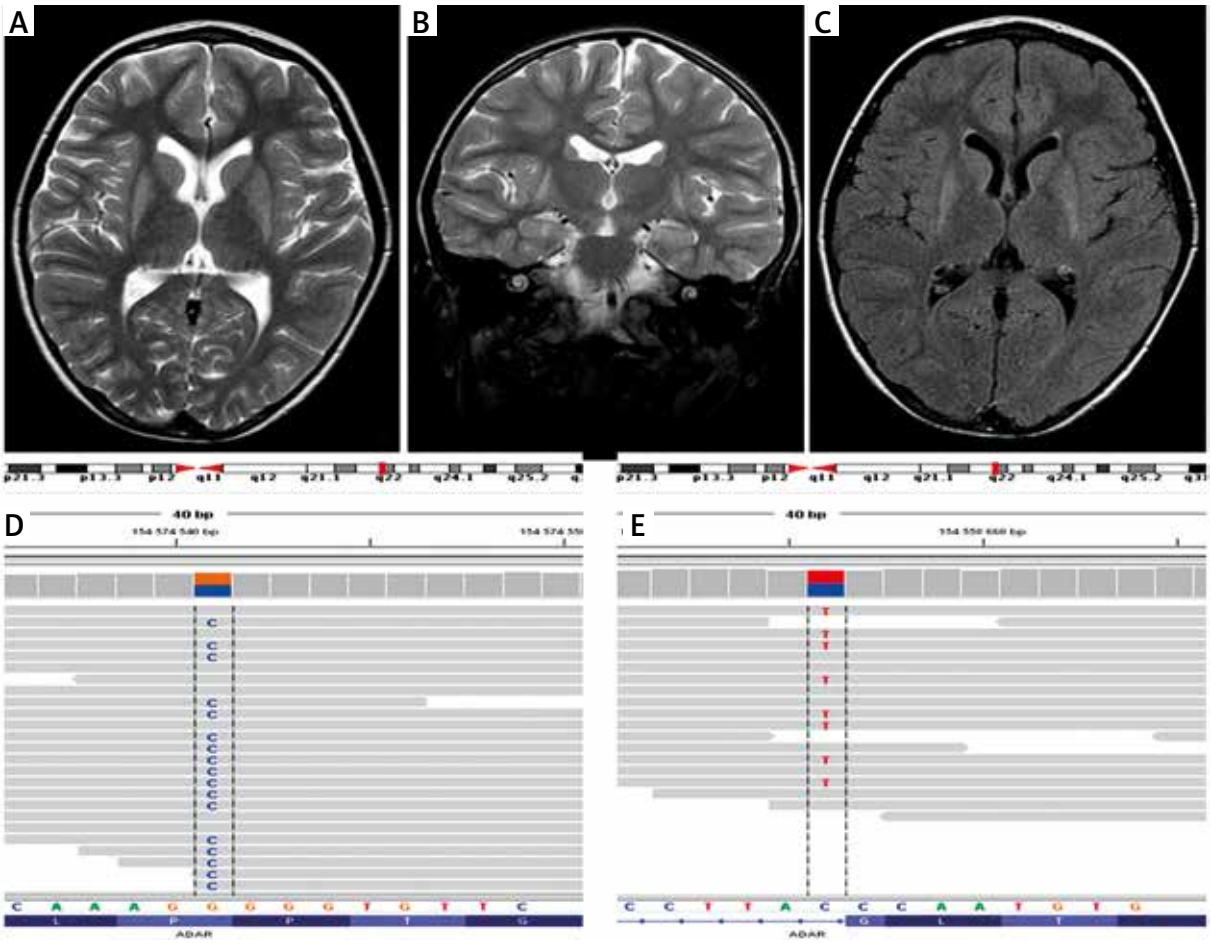

Fig. 1. Magnetic resonance imaging and molecular findings revealed in Case 1. **A-C)** Bilateral involvement of the putamen with hyperintense signal on T2-weighted images: axial plane, coronal plane, and on FLAIR sequence, respectively. **D, E)** Integrative Genomics Viewer picture of identified *ADAR* causative variants c.577C>G and c.3202+1G>A. The depth of coverage across the variants was 8/16 and 18/37, respectively

the highly evolutionary conserved z-alpha adenosine deaminase domain and results in removing important atomic interactions between protein and DNA/RNA [2].

Conclusions

In conclusion, we suggest that the disease should be always differentiated from LS to prevent diagnosis delay. We would like to underline that presence of specific MRI features of bilateral striatal necrosis and freckles-like skin changes should direct differential diagnosis to the *ADAR* mutations screening.

Acknowledgements

We thank the family for participation in the study. The study was partly supported by the CMHI project no. S136/13 and NSC grant no. 2012/05/B/NZ2/01627.

Disclosure

Authors report no conflict of interest.

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Folia Neuropathologica

Double origin of the superior cerebellar artery associated with homolateral haemorrhagic infarction of cerebellum

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Folia Neuropathol 2016; 54 (4): 410-417 DOI: 10.5114/fn.2016.64820

Abstract

The superior cerebellar artery (SCA) shows the least variable course and the lowest incidence of anatomical variations among cerebellar arteries. In the present study, an 84-year-old woman was affected by a cerebellar infarction which underwent haemorrhagic evolution in the following days. Neuroimaging investigations also showed a probable double origin of the left SCA. Neuropathological examination confirmed the presence of a large haemorrhagic infarction at the level of the superior portion of the left cerebellar hemisphere and vermis. The left SCA arose from two small arteries arising from the left aspect of the basilar artery and joining together after a course of 9 mm. Previous studies have reported the association of cerebrovascular pathologies, such as intracranial aneurysms, with fenestrations and double origins of the posterior inferior cerebellar artery. In the present case, the occurrence of an haemorrhagic infarction in the vascular field of an SCA with double origin is intriguing in suggesting a possible pathophysiological association.

Key words: *superior cerebellar artery, cerebellum, anatomical variation, stroke, xanthogranuloma, fenestration, angiography, magnetic resonance, computed tomography, neuroimaging.*

Introduction

The superior cerebellar artery (SCA) is the least variant among cerebellar arteries, showing by far the lowest prevalence of anatomical variations. However, the incidence of double SCA in magnetic resonance angiographies has been reported to go from 3% to 10% [8,24,26,27] and reports of triple SCA are also present in the literature [6,17,34]. The SCA may also arise from the proximal segment of the posterior cerebral artery, a pattern corresponding to a joint origin of the two vessels [24,27]. Fenestration of the SCA has also been rarely described [24,27] but to the best of our knowledge, angiographic or post-mortem reports of double origins of SCA are not available.

Thus, in this paper we present the first neuroradiological and neuro-pathological description of a double origin of SCA, which was also associated with haemorrhagic infarction of the corresponding cerebellar territory.

Case description

An 84-year-old woman underwent syncope with head trauma. A cerebral computed tomography (CT) performed in the local emergency department was negative, except for very small haematic hyperdensities in the cortex of the left insula and right parietal lobe. Neurological examination was also negative, apart from headache and one episode of vomiting.

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Cerebral CT was repeated the following day and, due to absence of significant changes, the woman was released. On these CTs, two calcified small masses, potentially ascribable to benign xanthogranulomas, were also visible at the level of the choroid plexuses of the lateral ventricles (Fig. 1).

In the following four weeks, the woman had progressive headache worsening and about after one month nausea and vomiting appeared again. Moreover, on the 35th day of clinical course, dizziness and balance disorders appeared and on the 39th day she was admitted again to hospital because of a fall without being able to get up. On admission, a cerebral CT showed large hypodensity in the left paravermian region of cerebellum, partially extending to the right paravermian region; in the context of the lesion, a hyperdense area was also present, which was ascribed to haemorrhagic infiltration (Fig. 2). A cerebral magnetic resonance (MR) on the 43rd day showed T2 flair hypersignal area in the territory of the left superior cerebellar artery, involving the homolateral middle cerebellar peduncle and the vermis; at the level of the superior vermis, a GE T2 hyposignal focus was also confirmed. Neuroradiologists made a diagnosis of subacute ischaemic lesion of the territory of the left superior cerebellar artery, with following modest haemorrhagic infarction. On the 46th day after syncope, CT angiography did not show vessel occlusions but identified duplication of the origin of the left superior cerebellar artery (Fig. 3). Meanwhile, the neurologic conditions of the woman worsened, with dysarthria, dysmetria, and ataxia. On the 55th day, a cerebral CT showed the evolution of the cerebellar lesion, which appeared as a voluminous hypodense area in the left cerebellum, with areas of intense contrast impregnation, partially imprinting the IV ventricle. The patient died on the 67th day of her clinical course.

Neuropathological examination was performed after fixation in 10% formalin. Examination of the vessels of the cerebral base showed mild asymmetry in the calibre of the vertebral arteries, with the left and right arteries showing diameters of 3.5 and 2 mm, respectively. The basilar artery had a diameter of 3.5 mm. The left SCA arose from two small arteries arising from the left aspect of the basilar artery, at

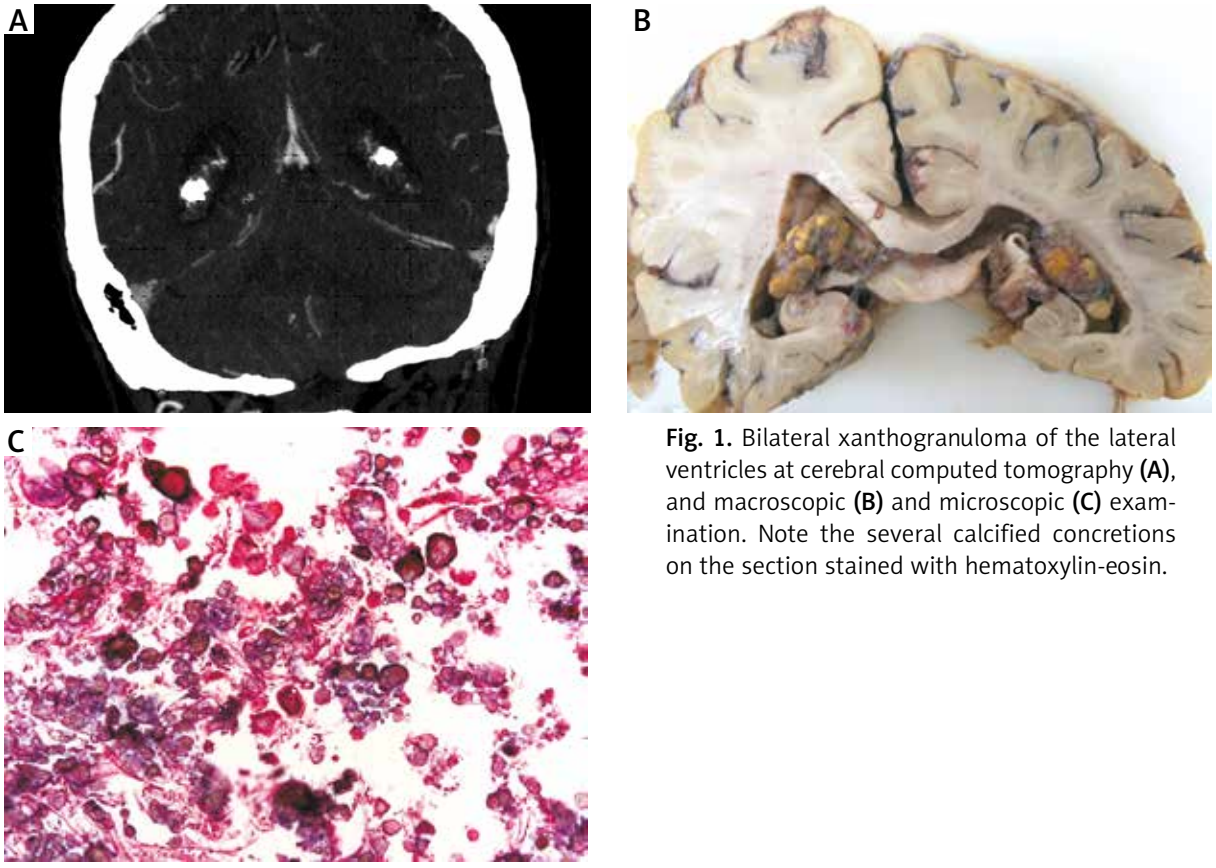

Fig. 1. Bilateral xanthogranuloma of the lateral ventricles at cerebral computed tomography **(A)**, and macroscopic **(B)** and microscopic **(C)** examination. Note the several calcified concretions on the section stained with hematoxylin-eosin.

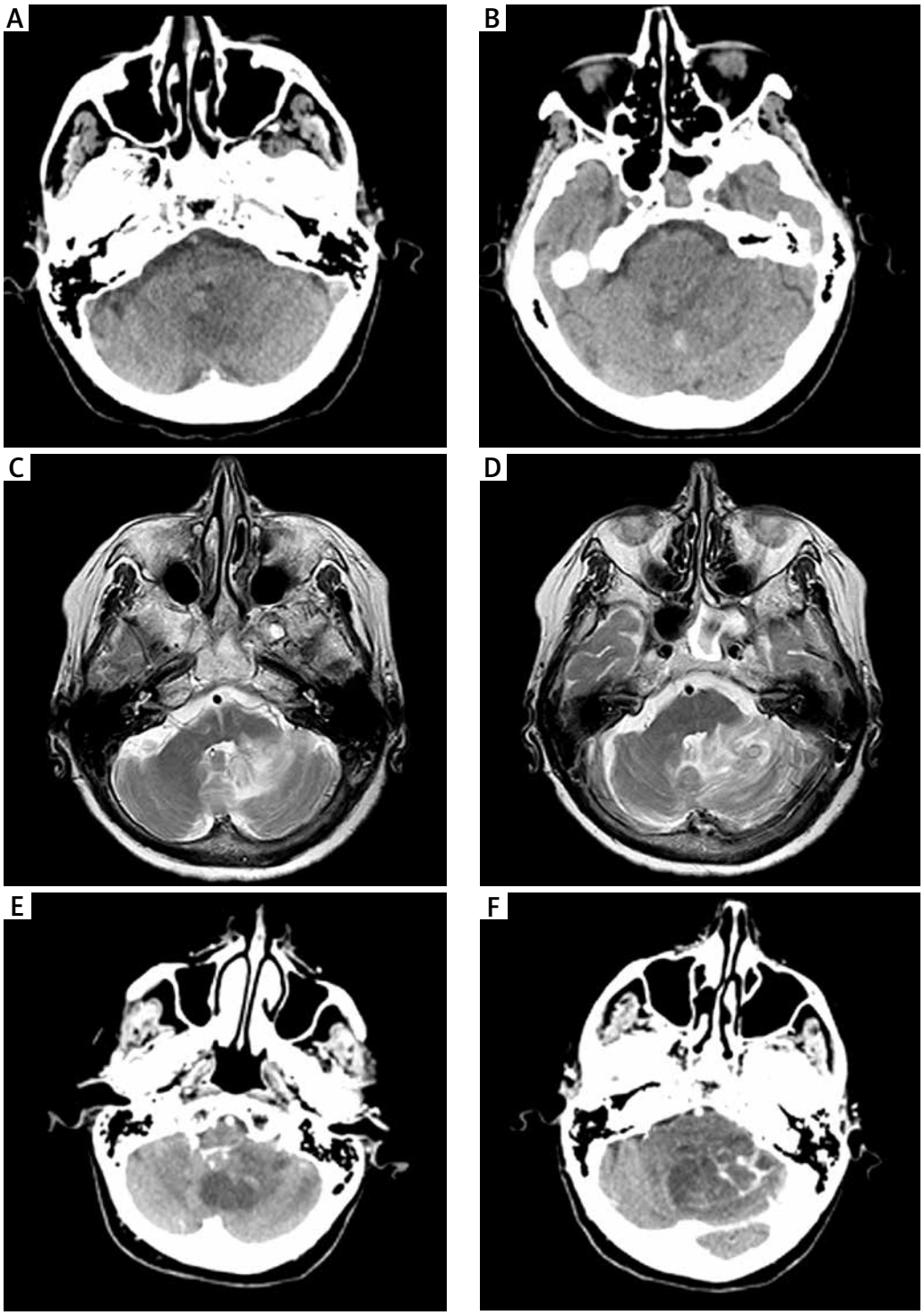

Fig. 2. A-B) Cerebral computed tomography (CT) on the 39th day of clinical course. Note the large hypodensity in the left paravermian region, partially extending to the right side, with a hyperdense area in its context. **C-D)** Cerebral magnetic resonance imaging (MRI) on the 43rd day showing T2 flair hyperintense area in the superior portions of the left cerebellar hemisphere, partially extending to the vermian and right paravermian region; a GE T2 hypointense area is also visible. **E-F)** Cerebral CT on the 55th day, more clearly showing the cerebellar lesion as a voluminous hypodense area with foci of intense contrast impregnation.

a reciprocal distance of 0.5 mm, and joining together after a course of 9 mm (Fig. 4A-B). Each vessel had an external diameter of about 0.5 mm along all of the course and up to the confluence; then, the SCA arisen from the two vessels showed a diameter of about 1 mm. A comparative analysis of the left SCA with the right SCA clearly showed that the two vessels giving the left SCA had smaller diameters than the right SCA, which showed a diameter never smaller than 1 mm.

Brainstem, still connected with cerebellum, was separated from cerebrum through a transverse section at the level of the mesencephalon. Macroscopic examination of the cerebellum showed bulging of the superior surface of the left hemisphere, with swelling of the cerebellar folia and partial homogenization of the nervous tissue (Fig. 4C). On the inferior aspect of cerebellum, a sulcus of tonsillar herniation was appreciable. Brainstem and cerebellum were cut together with sections parallel to the cerebellar circumference. In the left cerebellar hemisphere, an irregularly ellipsoid cavity of 3 x 2.5 x 1.5 cm, with necrotic-haemorrhagic content and anfractuous margins, was found. The lesion partially extended to the cerebellar vermis and the right paravermian region (Fig. 4D-E).

Section of the brain through multiple coronal planes showed two nodular lesions, with lobulated yellowish surfaces and firm consistence, at the choroid plexuses of the lateral ventricles (Fig. 1B).

Brainstem/cerebellum slices were paraffin embedded and sectioned. Histopathological examination confirmed the necrotic-haemorrhagic nature of the lesion (Fig. 4F) and revealed inflammatory leukocyte infiltrations of its walls. Thus, neuropathological

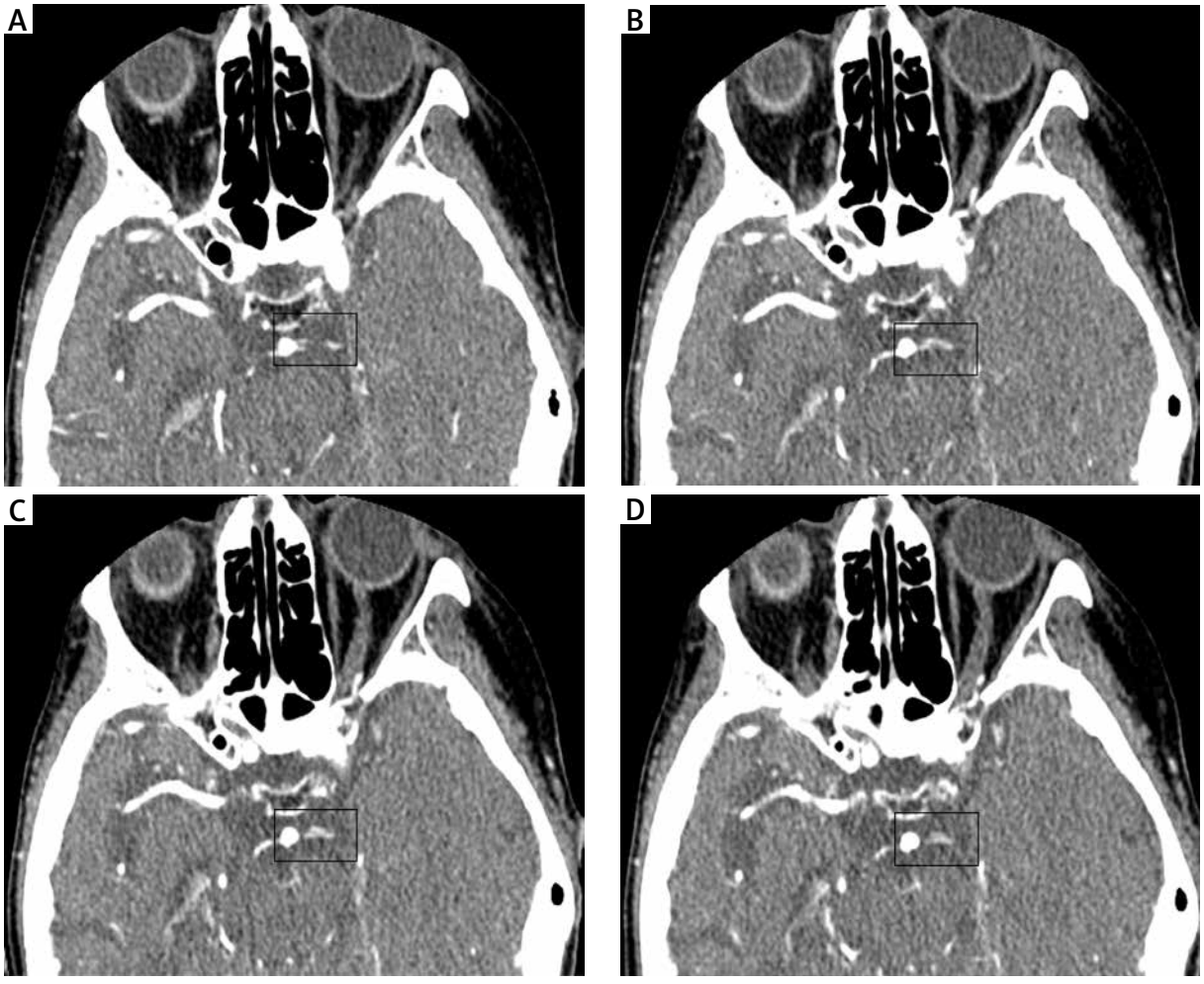

Fig. 3. Computed tomography angiography on the 46th day showing the two components of the superior cerebellar artery arising separately from the vertebral artery and converging after brief courses into a single vessel.

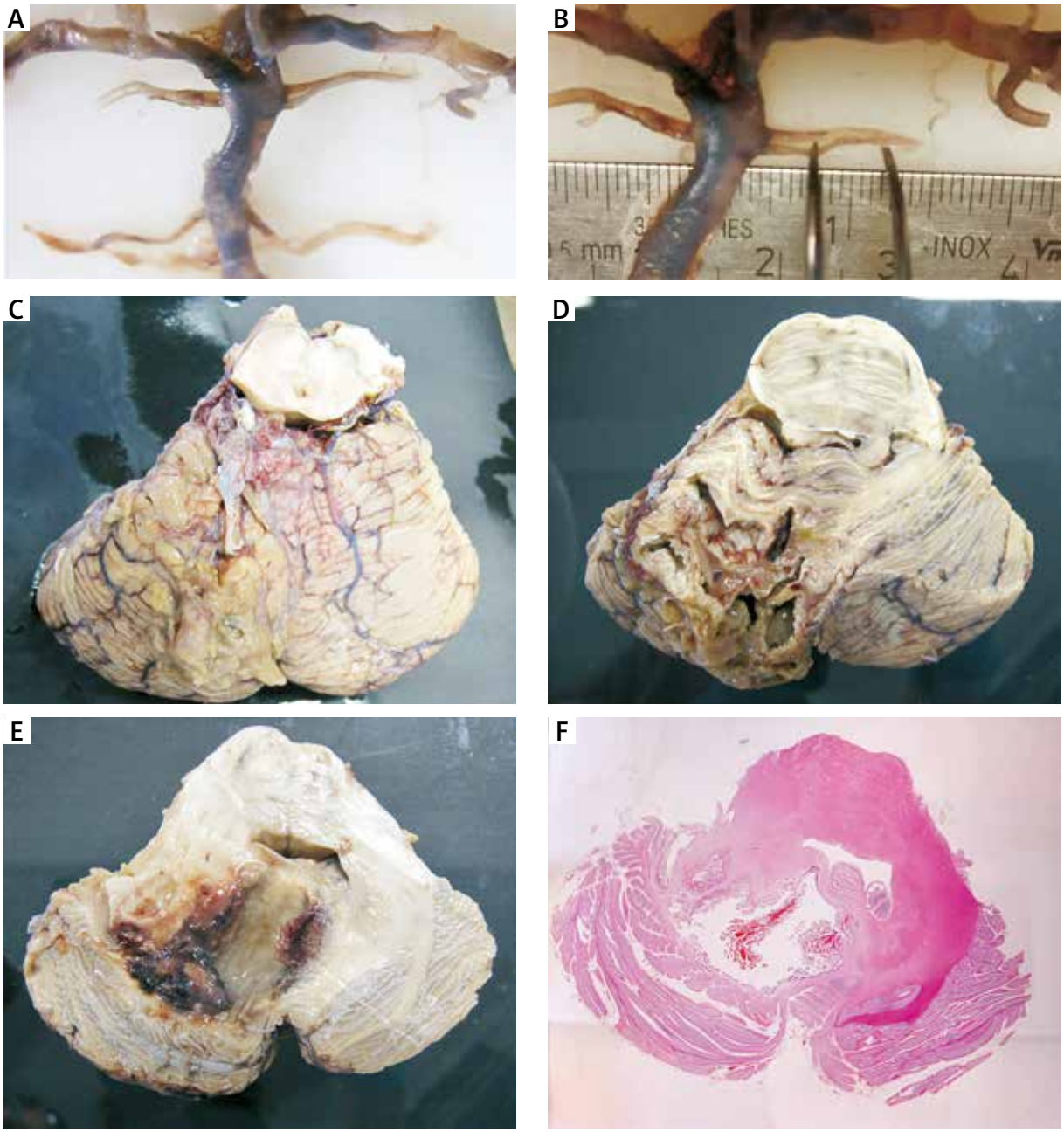

Fig. 4. A-B) Vision of the double origin of the superior cerebellar artery at macroscopic examination of the vessels of the brain base after their sampling. **C)** Superior surface of the cerebellum, showing bulging of the left cerebellar hemisphere, together with swelling and partial homogenization of the nervous tissue. **D-E)** Superior views of cerebellum after removing of the first and second slices in cranio-caudal progression. Note the large cavity with necrotic-haemorrhagic content and irregular margins. **F)** Cerebellar macro-section, corresponding to Figure E, at the level of the ischaemic lesion.

examination confirmed the neuroradiological diagnosis of subacute ischaemic lesion with haemorrhagic evolution of the left SCA territory. As regards the nodular structures in the lateral ventricles, multiple cholesterol clefts and several calcified concretions (Fig. 1C) were found in a context of granulomatous tissue, confirming the diagnosis of bilateral xanthogranuloma as incidental finding.

Discussion

To the best of our knowledge, this is the first report of double origin of SCA, which is usually considered the most constant cerebellar artery. The AICA also shows quite a low incidence of anatomical variations; fenestrations are very rare [28] and double origins of AICA are also not present in common databases of medical literature. The PICA, instead, is the most variable. PICAs with double origin have previously been reported in the literature both in angiography[1,3,10-12,14,15,19-21,29]andautopsy[22,32]. Fenestrations of the PICA have also been reported in few case reports [16,23] and contemporary presence of double origin and fenestration of one of the two components has been reported only twice [1,12].

The two components of a double origin of PICA usually arise at a certain distance to each other; Kwon *et al*. [10] have reported a distance between the two channels ranging from 19.5 to 35.6 mm, with the mean value of 24.9 mm. There are no descriptions or images of double origins of PICA with the two vessels at less than 1 mm to each other. Thus, the anatomical situation of the double origin of SCA described in the present paper is quite different, as in our case the two channels converging in the SCA arisen from the basilar artery at about 0.5 mm to each other.

The above difference between the two types of double origins may be a consequence of different morphogenetic mechanisms. The double origin of the PICA has been ascribed to persistence of anastomosis of PICA with the lateral spinal artery. In particular, the lateral spinal artery would become the caudal component of the double origin of the PICA while the rostral component would represent the PICA proper, embryologically derived from a hypertrophied radiculopial artery [11,14,15,21]. Some authors proposed that PICA fenestrations could result from partial regression of double origins [16]. Conversely, Lee *et al*. [12] have described the contemporary presence of double origin and fenestration as due to a hybrid of developmental variations, such as fenestration, duplication, abnormal regression and anomalous arterial origin. In the presented case of double origin of SCA, the abnormal persistence of an embryological vessel may also be hypothesized, although a very close relationship between the two vessels and the extreme rarity of the anatomical variation may not exclude alternative rarer mechanisms, such as for instance the division of an originally unique vessel.

Previous studies have reported the association of intracranial aneurysms with fenestrations and double origins of the posterior inferior cerebellar artery [1,14,15]. Aneurysms have been reported near the double origin of PICA or far away from the anatomical variation. In some cases, aneurysms have been specifically reported in the cranial or caudal channels of a double origin of PICA [9,10,13,19,20,29] or in the PICA after convergence of the two channels [9]. In particular, it has been suggested that double origin of the PICA could represent the effect of an underlying disorganization of the vascular development, which increases the risk of acquired intracranial aneurysms [15]. Some authors also recommended that a thorough search for intracranial aneurysms should be performed if a double origin of PICA is detected [21].

Some authors have also reported that one or both components of a double origin of PICA may be used as routes for endovascular treatments. In the cases when aneurysms are located in one of the two components of the double origin, the affected channel may be sacrificed through endovascular trapping, permitting preservation of blood flow through the other channel [10,13,19,20,29]. Only in one reported case such an approach has caused medullary infarction, probably due to occlusion of the perforating vessel at the tonsillomedullary segment [20], but balloon occlusion test has also been recently proposed for evaluation of the safety of the procedure [13]. We can consider that in a double origin as the one we described, endovascular access to one of the channels would probably be very difficult due to small calibres and close proximity of the vessels.

Apart from aneurysms, a pial arteriovenous fistula has also been reported at the level of a double origin of PICA, supporting the hypothesis of alterations in vascular development as a common pathogenetic mechanism [5]. Moreover, it must be considered that fenestrations of intracranial arteries have also been associated with other vascular anomalies, such as persistence of embryonal vessels, moyamoya disease and arteriovenous malformations [7,25,30,33].

In our case, aneurysms (or other of the above anomalies) were not present in the arteries of the brain base; however, bilateral xanthogranuloma of the choroid plexuses of the lateral ventricles coexisted. They are benign idiopathic lesions which are

usually asymptomatic and show an incidence of 1.6% to 7% of autopsies [18,31]. Their pathogenesis has not yet been clarified. Aging processes have been suggested, possibly correlated with hypercholesterolemia, atherosclerosis or diabetes mellitus, but an aberrant embryological development of the choroid plexus has also been proposed. In particular, xanthogranuloma may originally derive from neuroepithelial tubules (endowed of increased proliferative capacity) abnormally displaced in the stroma of choroid plexus during epithelial invagination [4]. Thus, also in our case, the coexistence of double origin of a cerebellar artery with another neuropathological entity possibly due to anomalous embryologic maturation of cerebral vascularization suggests the involvement of underlying developmental mechanisms.

Cerebellar haemorrhages may have particular characteristics with respect to other cerebrovascular districts. For instance, cerebellar small bleeds have been reported with higher frequency in cerebral micro-angiopathy due to hypertension and in progressive supranuclear palsy [2]. In the present case, the occurrence of a haemorrhagic infarction in the vascular field of an SCA with double origin is intriguing in suggesting a possible pathophysiological association. Although associated with a long-term compensation, the double origin of the left SCA may have represented an anatomic situation of haemodynamic instability, also due to the small distance between the two origins and the small diameters of the two vessels. Neuro-radiological and neuro-pathological investigations did not identify significant atherosclerotic disease nor possible sources of embolisms. Thus, it may be hypothesized that a hypotensive episode may have caused ischaemic damage to the territory of the left SCA, made more vulnerable due to the particular haemodynamic situation given by the double origin of the left SCA.

Disclosure

Authors report no conflict of interest.

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Folia Neuropathologica

Pyramidal signs in a Caucasian patient with spinal muscular atrophy: a case report

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Folia Neuropathol 2016; 54 (4): 418-421 DOI: 10.5114/fn.2016.64821

Abstract

Spinal muscular atrophy (SMA), an autosomal recessive disease, is characterized by the selective loss of spinal motor neurons due to reduced levels of the survival motor neuron (SMN) protein. The clinical symptoms of SMA are progressive proximal muscle weakness and paralysis. Here we describe a 20-year-old Turkmenistan male with SMA who presented with uncommon pathological reflexes and asymmetric onset of weakness. The diagnosis after genetic analysis revealed a homozygous deletion of SMN1 exons seven and eight. The copies of SMN2 exon seven were normal. Although pyramidal signs are not a common symptom of SMA, they could not be used to exclude the diagnosis of SMA in a patient with neuromuscular degenerative symptoms. Therefore, an additional attention is warranted to SMA patients with pathological reflexes.

Key words: *spinal muscular atrophy, pyramidal sign, amyotrophic lateral sclerosis.*

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by the selective loss of spinal motor neurons. This loss is due to reduced levels of the survival motor neuron (SMN) protein [10]. Spinal muscular atrophy is the leading genetic cause of infant mortality, and there are currently no effective treatments to slow progression of this disease [4,9]. Humans have two genes that produce SMN, *SMN1* and *SMN2*, the former of which is deleted or nonfunctional in the majority of patients with SMA. These two genes are nearly identical with one exception being a cytosine to thymine transition within exon seven of *SMN2*. This transition induces the exclusion of exon seven from 90% of the *SMN2* mRNA transcript. The truncated SMN protein is then rapidly degraded [7]. Consequently, most of *SMN2* mRNA transcripts are not able to compensate for the loss of *SMN1* in SMA patients. Thus, the *SMN2* copy number, proportional to the intact mRNA transcripts, is considered to be a modifying factor for the clinical severity of SMA [11]. The clinical symptoms of SMA are progressive proximal muscle weakness and paralysis. The differential diagnostic conditions include polymyositis and myodystrophy. Spinal muscular atrophy is clinically classified into four phenotypes based on the age of onset and motor function achieved. Spinal muscular atrophy type I has an onset of clinical signs before six months of age, type II between seven and eighteen months, type III between eighteen

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months and eighteen years, and type IV patients have adult onset [1].

Case report

A 20-year-old Turkmenistan male was hospitalized in March 2013 complaining of progressive limb weakness that he had experienced for the previous eight years. In otherwise good health, the patient suffered from weakening of the right lower limb muscles, which caused him to occasionally stumble, since 2005. In 2007, his left lower limb presented with similar symptoms that were at times accompanied by myoclonic jerks. The weakness in both lower limb muscles became progressively worse, which led to difficulties in walking and an inability to run and jump. Then in 2011, the patient developed weakening of both upper limb muscles, which did not affect his ability to write or move his upper limbs. The patient did not experience dysphagia or choking while drinking. The patient also had no family history of neuromuscular disease.

Upon physical examination, the patient was attentive and coherent and demonstrated normal hearing and vision. No atrophy or fasciculation of the tongue or facial weakness were observed. The patient exhibited normal strength of the sternocleidomastoid and cervical muscles and moderate muscular tension in his limbs. Neurological examination revealed muscle atrophy involving both the upper and lower extremities. The weakness was symmetrical and more proximal than distal, with the lower limbs generally being weaker than the upper limbs. A winged scapula and a pes cavus were observed. The patient exhibited a bilateral biceps strength of grade 5–, a triceps strength of grade 4, a bilateral iliopsoas strength of grade 2, and a grade 5– strength of the quadriceps, bilateral tibialis anterior and gastrocnemius. The grip strength of his hands was grade 5–. He had a Gower's sign and a waddling gait. Sensory function tests revealed no abnormalities. Deep tendon reflexes were tested and displayed a bilateral biceps reflex (+/++). The bilateral triceps reflex, Achilles tendon reflex and patellar tendon reflex were not elicited. The patient also had a bilateral Babinski's sign (Fig. 1) and Chaddock's sign.

Auxiliary examination showed the patient's creatine kinase (CK) levels to be 972 U/l, CK isoenzyme (CK-MB) levels to be 38 U/l, and lumbar pressure to be 224 mm H_2O. Routine cerebrospinal fluid tests, electrocardiogram (ECG), ultrasonic cardiogram and magnetic resonance imaging (MRI) examinations were unremarkable. Electromyography (EMG) indicated nerve damage on the bilateral first interosseous muscle, bilateral tibialis anterior, right quadriceps and bilateral rectus abdominis. A prolonged latent period was found in the cortex section of the deep sensory paths in the bilateral lower limbs. Both the lower limb motor evoked potentials had a decreased amplitude in the L4 nerve root. Muscle biopsy of the right biceps revealed angular atrophic fibers, and a nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) stain showed large fiber-type groups, demonstrating muscular "neurogenic" damage (Fig. 2). A multiplex ligation-dependent probe amplification (MLPA) kit using P021 probes was utilized to detect deletion and/or duplication of the *SMN1*/*SMN2* gene (Microbiology Research Centre, Holland, Amsterdam, The Netherlands). This examination discovered a homozygous deletion of *SMN1* exons seven and eight. The relative peak height ratios of *SMN2* exon eight increased to approximately 1.5, indicating that three copies of this exon were present. *SMN2* exon 7 was present at the normal copy number of two (Fig. 3). The patient was therefore diagnosed as having SMA type III based on the age of onset, disease progression and genetic test results.

Discussion

Spinal muscular atrophy is a severe neuromuscular disease that primarily involves the lower motor neurons in the spinal cord and brainstem, resulting in progressive muscular atrophy, fasciculation and decreased tendon reflex. The patient described here had the basic characteristics of SMA, however, he

Fig. 1. Evidence of the Babinski's sign.

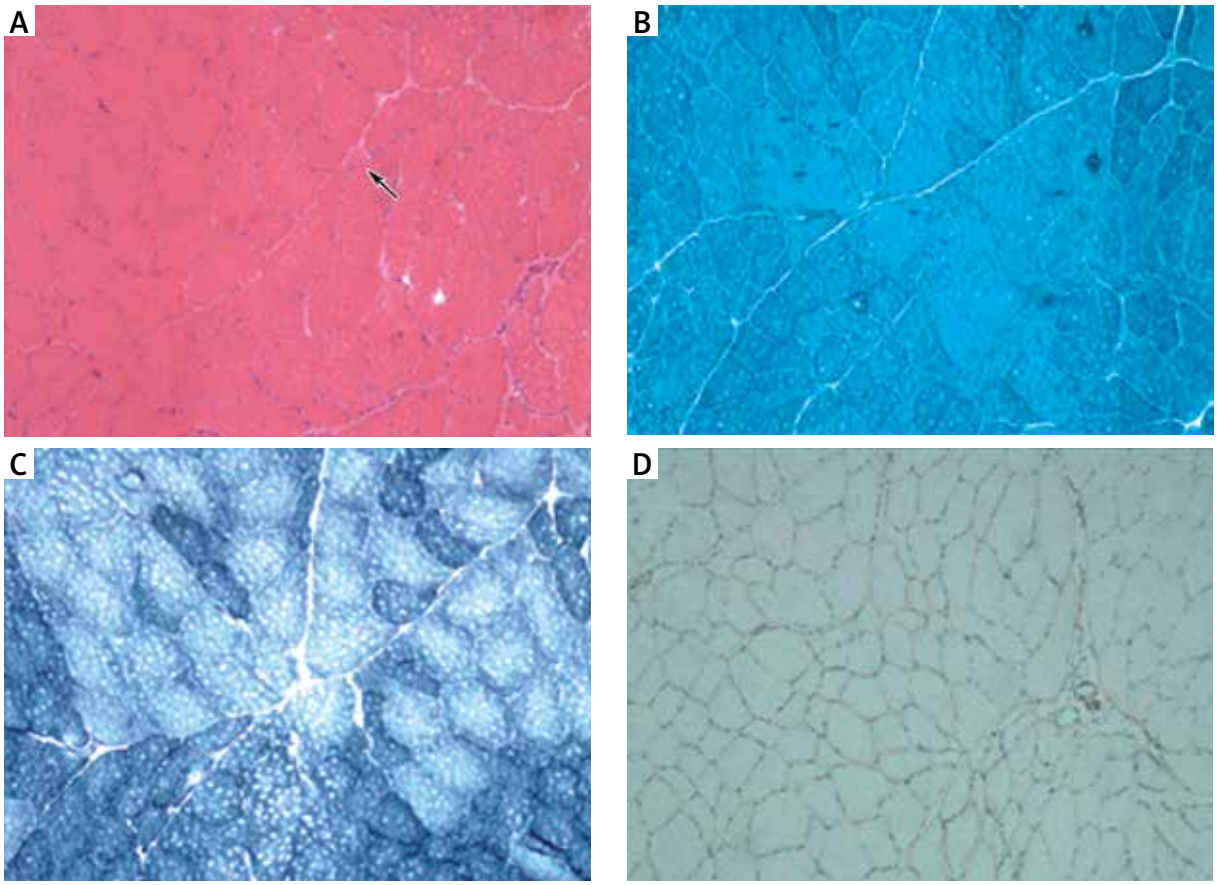

Fig. 2. Muscle biopsy of the right bicep (× 10). **A)** Hematoxylin and eosin staining reveals angular atrophic fibers (arrow). **B)** Gomori's trichrome staining indicates that there is no accumulation of abnormal materials. **C)** NADH-TR staining shows large fiber-type groups. **D)** The muscle positive stain dysferlin.

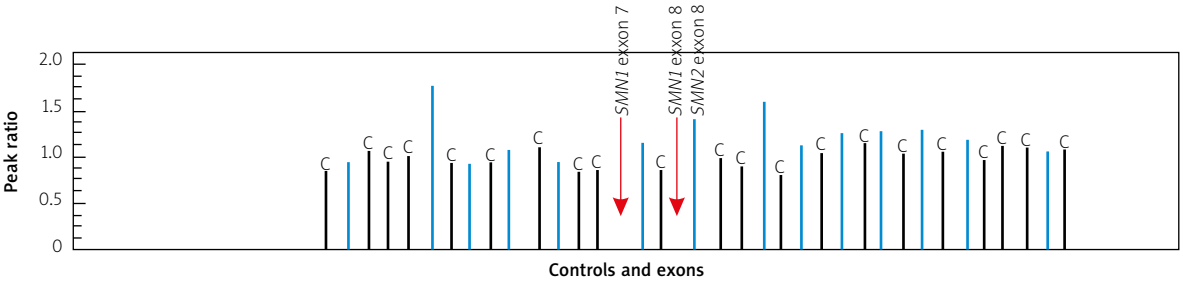

Fig. 3. Copy number analysis of *SMN1* and *SMN2*. The red arrows indicate homozygous deletions of *SMN1* exons seven and eight. The relative peak height ratio of *SMN2* exon eight increases to approximately 1.5, indicating the presence of three copies.

presented with pathological reflexes, which indicated an involvement of the pyramidal tract. There were no other etiologies to explain this rare condition. We had considered juvenile amyotrophic lateral sclerosis (ALS) during the procedure of diagnosis because the pathological reflex frequently indicates ALS. Amyotrophic lateral sclerosis, the most common and best-recognized form of motor neuron diseases, is characterized by a relentless degeneration of both upper and lower motor neurons leading to

progressive muscular paralysis [5]. Mutations in the superoxide dismutase (SOD1) and fused in sarcoma (*FUS*) genes are responsible for approximately 20% and 4-6% of familial ALS, respectively [12]. In the case presented here though, genetic tests confirmed the diagnosis of SMA-III and not ALS.

It is currently unknown whether SMA and ALS share molecular pathways in the process of motor neuron degeneration. As is common with neuromuscular degenerative disorders, the pathogenesis of ALS remains unclear. The multiple mechanisms thought to play a role in this disease include genetic factors, oxidative stress, excitotoxicity and protein aggregation, as well as impairments in RNA processing, axonal transport and mitochondrial function [2]. Synapses and distal axonal compartments are frequently involved in the early stages of ALS [6]. Mutations in *FUS* induce a redistribution of SMN from the axon to cytosolic FUS accumulations, which disrupt the function of SMN, thus leading to axonal defects [3]. Moreover, a study of 600 sporadic ALS patients found that an abnormal number of *SMN1* copies (one or three rather than two) occurred more frequently in cases than controls (OR = 2.8, 95% CI: 1.8-4.4) [8]. Since the SMN1 protein is ultimately affected in both ALS and SMA, a shared pathogenesis of these two disorders is very likely. Additional studies examining the molecular mechanisms involved in these two conditions should be performed.

No reports concerning pyramidal tract damage in Asian SMA patients exist. Symptoms of SMA in patients of various ethnicities should be further explored and compared to determine whether there are significant differences. In addition, patients with SMA typically present with symmetrical onset. One of the noteworthy characteristics of this case is that the patient's left lower limb experienced weakness two years after the right lower limb. This patient should be followed closely so that the differences in disease progression between this unique case of SMA and classical cases can be compared.

In conclusion, pyramidal signs cannot be used to exclude the diagnosis of SMA in patients with neuromuscular degenerative symptoms. While the cause of the SMA remains unclear, further investigation of SMA patients with pathological reflexes is necessary.

Disclosure

Authors report no conflict of interest.

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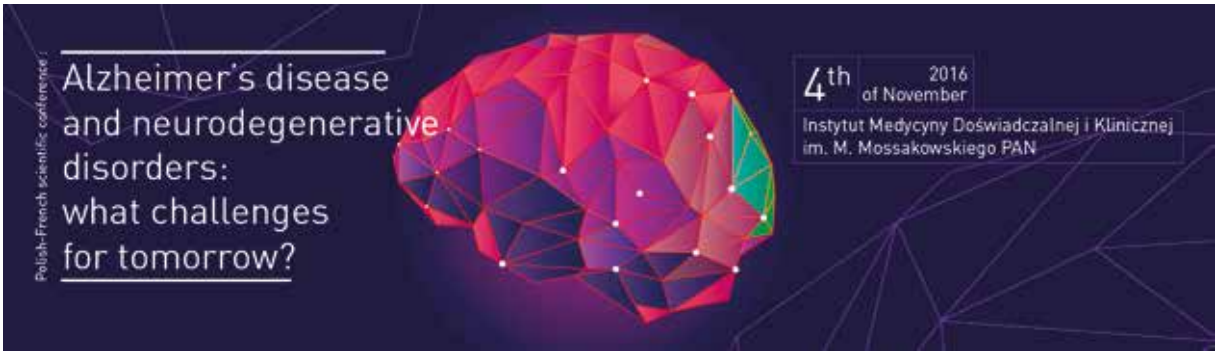

DOI: 10.5114/fn.2016.64824

Polish-French scientific conference:

Alzheimer's disease and neurodegenerative disorders: what challenges for tomorrow?

Mossakowski Medical Research Centre
of Polish Academy of Sciences

4th of November 2016

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

PREFACE

Polish-French conference "Alzheimer's disease and neurodegenerative disorders: what challenges for tomorrow?" was held at Mossakowski Medical Research Center of Polish Academy of Sciences on the 4th of November 2016. The event aimed to present the latest scientific achievements in the field of Alzheimer's disease and other neurodegenerative diseases. The conference was organized by **French Embassy in Warsaw, French Institute in Warsaw, Polish Academy of Sciences and Mossakowski Medical Research Center.** The conference was opened by Stanislas Pierret, Director of French Institute, Prof. Maria Barcikowska-Kotowicz, Director of Mossakowski Medical Research Center, and by Prof. Stanislaw Czuczwar, Vice-President of Polish Academy of Sciences. The invited guests were eminent specialists in the field of neuroscience and molecular biology. The professors lectures were given by French experts **Prof. Luc Zimmer** and **Prof. Philippe Corcia,** and Polish experts, **Prof. Tomasz Gabryelewicz** and **Prof. Konrad Reydak.** The professors lectures were complemented by oral presentations of young, talented and promising scientists: **Dr. Raphaëlle Pardossi-Piquard** and **Dr. Olivier Nicole** from France and **Dr. Michalina Wężyk** and **Dr. Anna Barczak** from Poland. Conference was attended by nearly 130 participants (#130 registrations, but a bit more than 90 people were there), including 23 poster presentations assembling various approaches and sectors in the study of Alzheimer's disease and neurodegenerative disorders. The conference was strengthening Polish-French cooperation in the field with an emphasis on young scientists networking. It is our hope that this conference will help in future scientific exchange and sharing valuable patient's derived material for the research. This is particularly important in the era of large-scale high throughput methods where single factors or single genes are no longer in the center of the study. There is an urgent need to join the forces and to conduct the research on thousands patients targeting entire gene conglomerates, the influence of multiple environmental factors and, consequently, whole genome, epigenome, and proteome that are the challenges for today and tomorrow's neurodegenerative research.

Dr. Michalina Wężyk, Mossakowski Medical Research Center
Dr. Sebastien Reymond, French Embassy in Warsaw
Dr. Antonin Borgnon, French Embassy in Warsaw
Organizing Committee

ORAL PRESENTATIONS

[1]

Toward PET molecular imaging of functional serotonin receptors during Alzheimer's disease

Prof. Luc Zimmer, PhD, DSc

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Evidence accumulates suggesting that drugs targeting the serotoninergic system could play an important therapeutic role in Alzheimer's disease (AD), particularly in terms of cognitive enhancement. Among the serotonergic targets, the 5-HTT1A and the 5-HT6 receptor subtypes are of particular interest to treat cognitive or non-cognitive symptoms in AD and several pharmaceutical and biotech companies are currently developing new drugs specifically targeting these receptors.

It is crucial that discovery and development of clinical candidate compounds are accompanied by parallel development of suitable positron emission tomography (PET) tracers to allow for a rational and robust testing of pharmacological hypotheses with neuroimaging. If PET is a powerful imaging modality mainly used to visualize and asses the distribution/density of targeted receptors in a living subject (animal, human), we propose that comparing PET imaging obtained using an agonist radiotracer, which binds selectively to functional receptors, with the PET imaging obtained using an antagonist radiotracer would provide original information on 5-HT receptor impairment during AD. This exploration of functional and active receptors at pre-dementia stages can open up the possibility of better pathophysiological understanding, differential diagnosis or assessment of the impact of pro-cognitive therapy.

[2]

Genetics of amyotrophic lateral sclerosis

Prof. Philippe Corcia, PhD, DSc

Centre de Ressources et Compétences SLA (CRCSLA), CHU Bretonneau, 2 Boulevard TONNELLE, 37044 Tours CEDEX9. Federation des CRCSLA Tours-Limoges LITORALS, Fellow de L'European Academy of Neurology, INSERM U 930, Université François-Rabelais de Tours, Faculté de Médecine, 10, Bd Tonnellé, 37032 Tours Cedex 1, France

Amyotrophic lateral sclerosis (ALS) is the most frequent motor neuron disorder in adulthood. This always fatal condition is characterized by upper and lower motor neurons degeneration in bulbar and spinal territories leading to death by respiratory failure after a median duration of 36 months. Although a huge literature clearly supports a major role of genetics in ALS, pathophysiology of ALS remains unknown. 10% of cases are familial and over the last 20 years, more than 25 genes have been linked to the disease, among which four (SOD1, TARDBP, FUS and mainly C9orf72 genes) explained more than 60% of familial (FALS) cases and around 10% of sporadic (SALS) cases. After an overview of recent findings of genetics in ALS, we will bring evidence that strongly support that ALS is a oligogenic affection and finally discuss whether genotype-phenotype correlations could be drawn in ALS. This clearly must be taken into account in clinical practice and more specifically in case of genetic counselling. In conclusion, genetics plays a key role in pathophysiology of ALS and probably will be the matter of promising clinical trials in the next years.

[3]

C99 as an early contributor to AD pathology

Raphaëlle Pardossi-Piquard[1], Inger Lauritzen[1], Alexandre Bourgeois[1], Sophie Pagnotta[2], Maria-Grazia Biferi[3], Martine Barkats[3], Pascale Lacor[4], William Klein[4], Charlotte Bauer[1], Frederic Checler[1]

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Endosomal-autophagic-lysosomal (EAL) dysfunction is an early and prominent neuropathological feature of Alzheimers's disease, yet the exact molecular mechanisms contributing to this pathology remain undefined. By combined biochemical, immunohistochemical and ultrastructural approaches, we demonstrate a link between EAL pathology and the intraneuronal accumulation of the β-secretase-derived βAPP fragment (C99) in two *in vivo* models, 3xTgAD mice and adeno-associated viral-mediated C99-infected mice. We present a pathological loop in which the accumulation of C99 is both the effect and causality of impaired lysosomal-autophagic function. The deleterious effect of C99 was found to be linked to its aggregation within EAL-vesicle membranes leading to disrupted lysosomal proteolysis and autophagic impairment. This effect was Aβ independent and was even exacerbated when γ-secretase was pharmacologically inhibited. No effect was observed in inhibitor-treated wild-type animals suggesting that lysosomal dysfunction was indeed directly linked to C99 accumulation. In some brain areas, strong C99 expression also led to inflammatory responses and synaptic dysfunction. Taken together, this work demonstrates a toxic effect of C99 which could underlie some of the early-stage anatomical hallmarks of Alzheimer's disease pathology. Our work also proposes molecular mechanisms likely explaining some of the unfavorable side-effects associated with γ-secretase inhibitor-directed therapies.

[4]

Behavioral and electrophysiological characterization of the learning and memory deficits induced in mouse model of Alzheimer's disease

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Post-learning hippocampal sharp wave-ripples (SWRs) generated during slow wave sleep are thought to play a crucial role in memory formation. While in Alzheimer's disease, abnormal hippocampal oscillations have been reported, the functional contribution of SWRs to the typically observed spatial memory impairments remains unclear. These impairments have been related to degenerative synaptic changes produced by soluble amyloid beta oligomers (Aβos) which, surprisingly, seem to spare the SWR dynamics during routine behavior. To unravel a potential effect of Aβos on SWRs in cognitively-challenged animals, we submitted vehicle- and Aβo-injected mice to spatial recognition memory testing. While capable of forming short-term recognition memory, Aβ mice exhibited faster forgetting, suggesting successful encoding but an inability to adequately stabilize and/or retrieve previously acquired information. Without prior cognitive requirements, similar properties of SWRs were observed in both groups. In contrast, when cognitively challenged, the post-encoding and -recognition peaks in SWR occurrence observed in controls were abolished in Aβ mice, indicating impaired hippocampal processing of spatial information. These results point to a crucial involvement of SWRs in spatial memory formation and identify the Aβ-induced impairment in SWRs dynamics as a disruptive mechanism responsible for the spatial memory deficits associated with Alzheimer's disease.

[5]

Apraxic variant of Alzheimer's disease – case presentation

Anna Barczak, PhD

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Corticobasal syndrome (CBS) is increasingly reported in subjects with confirmed Alzheimer's disease underlying pathology. With the exception of memory problems, investigations point to severe apraxia, slowly progressive left hemi-Parkinsonism, myoclonus, visuospatial disturbances, resembling Corticobasal degeneration (CBD). 55 yo female with problems in occupational and everyday activities, reporting impairment in memory, visuospatial domains as well as in writing, speech and calculation was hospitalised with initial diagnosis of CBD. Severe dementia (MMSE 14) was diagnosed, with parkinsonism and dysfunctions in learning, episodic memory, executive functions along with dispropor-

tionally pronounced apraxia, spatial disorientation, writing, speech, mathematical skills dysfunctions. Neuroimaging examination revealed the presence of multiple small vascular changes in frontal white matter and ventricular areas together with the cortical atrophy in the frontal and parietal lobes, but hippocampal regions were intact. CSF-AD biomarkers profile with decreased level of beta-amyloid and increased levels of total and phosphorylated tau proteins was characteristic for Alzheimer's disease. Final diagnosis was AD-CBS plus syndrome and despite donepezil treatment, her condition was rapidly worsening, with psychotic features and complete loss of the independency. After 3 years of observation the MMSE = 0, subject is mute, presents motor problems and requires full care.

[6]

DNA damage stress response in Alzheimer's disease

Michalina Wężyk[1,2], Aleksandra Szybińska[1], Marcelina Szczerba[1], Joanna Wojsiat[3], Mariusz Berdyński[1,6], Beata Pepłońska[1], Jakub Fichna[1], Jan Ilkowski[4], Maria Styczyńska[5], Marzena Zboch[6], Anna Filipek[5], Magdalena Skrzypczak[8], Krzysztof Ginalski[8], Michał Kabza[9], Izabela Makałowska[9], Urszula Wojda[3], Maria Barcikowska[1,5], Cezary Żekanowski[1]

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Whole-transcriptome profiling of primary cell lines of fibroblasts derived from fEOAD patients with mutations in PSEN1 revealed disturbances in signaling pathways linked with regulation of cell cycle and DNA damage response (DDR). The transcriptomic data were further subjected to functional validation in terms of DDR. We found disturbed activity of ATR and ATM kinases, expressed by their activation status and abnormally increased phosphorylation of their downstream effectors, Chk1 and Chk2 kinases. These effects were accompanied by an increased phosphorylation of BRCA1 at Ser1524 in fEOAD cells. Simultaneously, we have observed a drop in nucleic BRCA1 (Ser1524) level in fEOAD, suggesting disturbances in translocation of BRCA1 to nucleus. Abnormal subcellular localization of phosphorylated BRCA1 could interfere with DNA repair and redirect the cells to apoptosis that was found to occur at a greater extent in fEOAD patients than in control cell line, what was demonstrated by an increase of percentage of apoptotic cells using flow cytometry and by the content of nuclear enzyme PARP cleaved by caspase 3 to fragments of 89 and 24 kDa. We concluded that BRCA1 may influence presenilin 1 reprocessing and this opens very interesting novel research area linking Aβ42-based pathology in familial AD with PSEN1 mutations and possibly pathology in sporadic AD, suggesting that BRCA1-targeted treatment could work for both types of AD.

This work is supported by Polish National Science Centre grant "SONATA6" no. G11192013/09/D/NZ3/01348 and Statutory Grant at MMRC from Ministry of Science and Higher Education.

[7]

A project within the EU Joint Programme for Neurodegeneration Biomarkers for Alzheimer's disease and Parkinson's disease

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The main goal for the BIOMARKAPD project was to standardize the sampling and measurement for the already known biomarkers, as well as to develop new ones for AD and PD, and standardize clinical use biomarkers. This has been done by developing and validating protocols for these

processes and to give training courses for the staff. As a result, most of the centers in Europe are performing this procedure in a common, standardized way. The protocols for analysis of CSF Aβ, P- and T-Tau (AD markers) has been done by developing both for clinical practice and for clinical trials. Some scientific highlights of this project consists; (1) The largest subject-level meta-analysis on the prevalence of amyloid abnormality in non-demented subjects was performed; (2) A novel ELISA for neurogranin, a dendritic marker, was validated clinically; (3) A novel fully automated method for CSF Aβ42 measurement was validated; (4) A capillary isoelectric focusing immunoassay for Aβ fragments was developed and validated. Some structural highlights consists: (1) Connecting researchers who work on biomarkers for Alzheimer's and Parkinson's diseases around Europe; (2) The partners set-up a central and virtual biobanks and 5 litres of CSF has been collected; (3) The reference method developed in BIOMARKAPD will now be used for CSF Aβ42 T-tau and P-tau.

POSTERS

[1]

Basal forebrain cholinergic neurons morphology in mouse models of Alzheimer-type and frontotemporal dementia-type tauopathy

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Loss of basal forebrain cholinergic neurons (BFCN) is a hallmark of the Alzheimer's disease (AD) thought to contribute to the cognitive dysfunctions. To this date, the mechanisms underlying cholinergic neurons degeneration remain uncertain. The present study aimed to investigate the relationship between tau neurofibrillary degeneration and cholinergic defects of two tauopathic mouse models: Line 1 (L1), with mild Alzheimer's disease-like tauopathy and Line 66 (L66) with severe frontotemporal lobar degeneration-like tauopathy (FTLD). Experiments were carried out on 3- and 9-month-old animals. Immunohistochemical stainings were performed for cholinergic markers ChAT and p75NTR. BFCNs were less numerous and showed lower expression of ChAT and p75NTR in L1-AD mice, but not in L66-FTLD mice as compared to wild type NMRI mice. The impairments in L1 were observed in interneurons of striatum as well as in projection neurons in medial septum, vertical and horizontal limb of diagonal bands and magnocellular basal nucleus of both age-groups. In summary, obtained results may suggest a loss of cholinergic phenotype or even neuronal deaths in L1-AD animals as early as in 3 month of age with no change in function in L66-FTLD animals at the same age.

This work was founded by NCN grant 2014/15/B/NZ4/05041.

[2]

The point mutations of APP gene (Amyloid beta precursor protein) as a target for SNP-selective RNA degradation by ribonuclease H, using modified antisense oligonucleotides

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Amyloid β precursor protein is an integral membrane protein and occurs especially abundant in the synapses of neurons. It undergoes regulated intramembrane proteolysis that generates beta amyloid polypeptide (Aβ) whose fibrillar form plays a major role in the pathogenesis of Alzheimer's disease. Exons 16 and 17 of the APP gene encode the Aβ peptide. Point mutations of these exons affect metabolism and stability of the Aβ, being the cause of some percentage of familial, dominant AD. In the presented study, three cases of familial, dominant AD-causing mutations: London (V717I), Flemish (A692G) and Arctic (E693G) were investigated as a targets for allele-selective RNA degradation by cellular ribonuclease H. Two different and new approaches using modified antisense oligonucleotides were applied. In total, about 90 antisense oligonucleotides, carrying different nucleotide and non-nucleotide modifications, were tested to activate RNase H to selectively cut the mutant RNA without affecting the wild type form. The highest selectivity of degradation was observed for Flemish RNA variants, that include C-to-G nucleotide substitution. Among 44 designed oligonucleotides to this particular case, 20 caused selective degradation of mutant RNA variant in in vitro assay, but only 8 differentiated the alleles ratio in HeLa cells.

[3]

Purinergic P2X7 receptor is involved in alpha-synuclein-mediated neuronal cell death via dysregulation of PI3K/AKT and AMPK-mTOR signaling pathways

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In parallel to the alpha-synuclein (ASN) hypothesis of Parkinson's disease (PD) aetiology, purinergic receptors have been recently identified as mediators of Lewy bodies accumulation and dysregulation of dopaminergic neurotransmission. However, the mechanisms underlying the disturbances in purinergic signalling in PD are not well established. The aim of our study was to investigate the role of purinergic receptors in mechanisms of ASN-evoked cell death. As a research model we used neuroblastoma (SH-SY5Y) cell line as well as rat synaptoneurosomes treated with exogenous soluble ASN. We observed that exogenous ASN activates P2X7 receptor (P2X7R) resulting in calcium influx and pannexin channel-dependent ATP release. Treatment with either non-selective (PPADS) or selective (AZ11645373) P2X7R antagonist prevented the elevation of cytosolic calcium as well as cytotoxicty evoked by extracellular ASN. We identified that downstream intracellular signalling of ASN induced cytotoxicity implicate two signalling pathways: P2X7R-PI3K/AKT and P2X7R-AMPK-mTOR axis. When exposed to exogenous ASN, overactivation of P2X7R perturbs the balance between those pathways leading to concurrent blockade of the mTOR signalling and neuronal cell death. Our study provide new insight into our knowledge of the relationship between purinergic signalling and ASN and provides a further rationale for anti-purinergic therapy of synucleinopathies.

Supported by NSC grant 2013/09/D/NZ3/01359.

[4]

Cholinesterases inhibition of new tacrine-melatonin heterodimers

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Alzheimer's disease (AD) is the most common form of neurodegenerative dementia affecting elderly and middle-aged people. Cholinesterases (ChE) – acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are acetylcholine (ACh) hydrolyzing enzymes and their inhibition still represents the currently employed approach for the treatment of AD.

As a continuation of our studies, we designed compounds with inhibitory activity against cholinesterases. In this study we report biological activity of several series of hybrid molecules – heterodimeric compounds combining substituted tacrine with a melatonin or cyclic melatonin derivative.

The cyclic derivative – structurally similar to physostigmine – highly improve the neurotransmission of acetylcholine.

The activity of the synthesized compounds was evaluated with spectrophotometric Ellman's method. This method of evaluation of the activity of cholinesterase inhibitors is based on the fact that AChE/BuChE interacts with the inhibitor, thus preventing the acetyl/butyrylcholine hydrolysis. The inhibitor diminishes the enzyme activity which is dependent on the inhibitor concentration.

The new compounds of these novel hybrids exhibit inhibitory activity against cholinesterases, especially BuChE, and can be used as an important starting point for further investigation of the possible use of these compounds in the therapy of neurodegenerative diseases.

This work was supported by the National Science Center Grant DEC-2011/03/B/ST5/01593.

[5]

Discrimination between Alzheimer patients and controls by means of EEG connectivity measures

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Alzheimer disease (AD) instrumental assessment includes biomarkers based on amyloid β and tau proteins in cerebrospinal fluid (CSF), MRI and PET. These procedures are expensive and not widely available (PET, MRI) or invasive (PET, CSF). A promising cheap, largely available, repeatable and non-invasive technique is electroencephalography (EEG). AD deteriorates neuronal networks, so functional brain connectivity as estimated from EEG was hypothesized to discriminate between AD and normal elderly (Nold) individuals.

Resting state eyes-closed EEG data were recorded (19 electrodes of 10-20 system) in 42 Nold and 42 AD subjects with dementia. The connectivity measures: coherences and Directed Transfer Functions (DTF – a measure of directed signal propagation in brain) were analyzed in delta, theta, alpha, beta and gamma bands.

Compared to Nold group, AD showed decrease of EEG coherence and posterior-to-anterior decrease of propagation as revealed by DTF, especially at theta (4-8 Hz) and alpha (8-13 Hz) bands. The features best discriminating between both groups were determined by means of statistical tests and principal components analysis. The classification yielded sensitivity = 86%, specificity = 70%. Including alpha frequency peak into the classification parameters resulted in sensitivity = 90% and specificity = 0.67%. These results indicate promising perspectives of AD assessment with EEG.

Supported by FP7 project DECIDE, www.eu-decide.eu

[6]

Periodontitis and periopathogens as a risk factor for Alzheimer's disease and stroke

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Studies have shown that systemic, peripheral infections affect AD patients. Chronic infection can cause slow progressive dementia and cortical atrophy.

Emerging evidence suggests that poor oral health influences the initiation and/or progression of diseases such as atherosclerosis (including myocardial infarction and stroke), diabetes mellitus and neurodegenerative diseases. Periodontal disease (PD) is a common chronic infectious disease often resulting in tooth loss. An inflammatory response in the periodontal tissues is caused by microorganisms present in dental plaque. Specific bacterial ligands increase the expression of proinflammatory molecules, which activates the innate and adaptive immune systems. Evasion of pathogens from destruction by the host immune reactions leads to persistent infection, chronic inflammation, neuronal destruction and Aβ deposition. Aβ has been shown to be a pore-forming antimicrobial peptide, indicating that Aβ accumulation might be a response to infection.

We started epidemiological studies on a prevalence of PD among Polish AD and post-stroke patients. The first step was focused on a periodontal status analysis in a cohort of 120 patients after (within 72 hours) hemorrhagic and non-hemorrhagic stroke. We found significant differences in the BOP and API values between patients and control subjects which confirm that PD can be predisposing factor to stroke.

[7]

Plasma levels of hsa-miR-107-5p and hsa-miR-650-5p in relation to APOE genotypes in Alzheimer's disease patients

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Background and aims: Alzheimer's disease (AD) is characterized by brain deposition of amyloid-β (Aβ). Apolipoprotein E (APOE) may be involved in Aβ clearance. There are 3 common APOE variants: E2, E3 and E4. The miR-650 probably regulate the expression of APOE. The miR-107 was shown to influence amyloid cascade and is downregulated in AD. The aim of the study was the analysis of relative levels of miR-107 and miR-650 in plasma of AD patients and in control group in relation to APOE genotype.

Material and methods: We investigated 35 AD patients, 42 control subjects and 37 persons in comparative group. The plasma levels of miR-107 and miR-650 were measured by qPCR and normalized per external standard – cel-miR-39-3p.

Results: Preliminary study has shown that the miR-107 was insignificantly decreased in plasma of AD patients and the level of miR-650 was increased in AD patients as compared to controls. In comparative group the normalized relative level of miR-107 was higher than miR-650, in AD patients the relation was reversed. Moreover, the APOE E4 genotype was correlated with decreased levels of both miR-107 and miR-650.

Conclusions: It seems that miR-107 and miR-650 may be associated with pathogenesis of AD, mediated by APOE gene.

[8]

The organization of health care for patients in daily care centers for people with dementia and quality of life of caregivers

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The research was devoted to the issues of organization of patient carewith dementia in Poland in the context of assessing quality of life for those who care.

Both in Poland and the world is still growing number of older people, so we should be interested in problems of people experiencing their own age and to move away from stereotypes defining seniors peripheral social position. In Poland, in the absence of sufficient professional, institutional forms of support for this group of patients, most commonly the entire burden of care falls on family carers. Long-term care for a person with dementia creates multiple load, among which the most important are: psychological stress, physical, economic and social. The main purpose of the research study was to assess whether there is a link between the organization of health assistance (understood as home care vs. institutional care, when the patients spend time in daily care centers) and the quality of life of carers. The data obtained allowed to determine the direction and intensity of the relationship between psychosocial variables and quality of life in both groups were similar and differed among themselves.

[9]

Assessment of sensitivity biomarkers for the determination of Amyotrophic Lateral Sclerosis (ALS) progress

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Amyotrophic lateral sclerosis (ALS) is one of the progressive neurodegenerative diseases of upper and lower motoneurons, leading to death within 3-5 years (with a median survival of 3 years from the symptom onset), mainly because of respiratory inefficiency and hypoxemia. ALS occurs either in familial (fALS) or, more frequently, in sporadic forms (sALS). Approximately 2 per 100,000 people worldwide are affected every year. The aetiology of ALS remains still unclear [Folia Neuropathol 2011, 49(1): 1-13]. Currently, effective treatments for ALS are not available, hence the discovery of sensitive biomarkers for the disease activity can offer tools for the rapid diagnosis and provide some insights into the pathophysiology of ALS, as well as for new therapeutic strategies [Transl Neurodegener 2015, 4: 17]. The present study demonstrates evaluation of the sensitivity, the specificity and relations of erythropoietin (EPO), some metalloproteinases (MMPs), as well as their tissue inhibitors (TIMPs) levels, in sALS patients with mild and severe symptoms. The results of this analysis and our previous clinical studies [J Neural Transm 2010, 117(3): 343-7; Eur J Neurol 2010, 17(2): 226-31] suggest the discriminative and the prognostic potential of CSF EPO and MMP-2 as biomarkers for the recognition/monitoring of ALS progress.

Authors thank Dr Piotr Janik for his neurological consultation in ALS patients and valuable remarks. This study was supported by the statutory budget of the MMRC PAS (Z-526, topic 23). Computations and analyses were carried out using the computational infrastructure of the Biocentrum – Ochota project (POIG.02.03.00-00-0030/09).

[10]

The combination of mass spectrometry and fluorescent methods to study amyloid-beta peptide aggregation inhibition

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Amyloid-β peptide (AB) varies in length from 39 to 43 amino acids and is generated during degradation of amyloid precursor protein. It is believed this peptide is involved in the development of Alzheimer's disease (AD) but its pathological role is not fully understood. To understand the amyloid-β peptide role in Alzheimer's disease a lot of

analytical tools have been proposed, among others mass spectrometry and fluorescent methods.

In our lab we decided to use the combination of these two methods to study AB (1-43) aggregation inhibition in the presence of protein hydrolysates derived from different sources. For this purpose, the AB was incubated in Tris buffer and the progress of aggregation was monitored by triple quadrupole mass spectrometer in multi reaction monitoring mode (MRM) as well as by spectrofluorometer.

Results obtained in the project "Diet supplement in prevention of neurodegenerative diseases" supported by The National Centre for Research and Development (Poland) in the program INNOTECH II, contract number INNOTECH-K2/IN2/68/183055/NCBR/13.

The study was carried out at the Biological and Chemical Research Centre and at the Faculty of Chemistry, University of Warsaw, established within the project co-financed by European Union from the European Regional Development Fund the Operational Innovative Economy, 2007-2014 and 2007-2013, Priority 2., Infrastructure R&D, Support for development of research infrastructure of scientific institutions implemented under the financing agreement No. POIG.02.02.00-14-024/08-00, dated 08.10.2009.

[11]

Oligomeric states of Human Cystatin C variants – atomic force microscopy and spectroscopic studies

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Human Cystatin C (hCC) is a small protein consisting of 120 amino acids that belongs to the type 2 cystatin family, which function is the inhibition of papain- and legumain-like proteases [1]. What is interesting, this protein is fully active in monomeric form, but was observed in dimeric and oligomeric states as well as fibrils.

Hereby we present a study conducted to obtain and characterize high molecular weight oligomers from wild type hCC as well as oligomers of hCC variants with single-point mutations, particularly concerning the residues 68^{th} and 57^{th}. The first is the location of naturally occurring mutation (L68Q) leading to hereditary cystatin C amyloid angiopathy (HCCAA-I) and the second mutation is responsible for the conformational instability leading the dimer formation [2]. Both, oligomers and fibers, were visualized by AFM and TEM techniques. Additionally, we assessed the secondary structure content for all studied proteins using infrared spectroscopy.

This research project has been financed by the funds from the National Science Centre (Poland) granted on the basis of decision no. DEC-2012/06/M/ST4/00036.

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[12]

Studies on budding yeast Hsp31p protein orthologous to Parkinson's disease-associated DJ-1 and to *Candida albicans* Glx3

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Saccharomyces cerevisiae Hsp31p belongs to a DJ-1/ThiJ/PfpI family together with human DJ-1 protein, whose mutations are implicated in hereditary early onset Parkinson's disease and with Glx3 protein that may be involved in *Candida albicans* pathogenicity. Hsp31p was previously shown to be important for survival in the stationary phase of growth and under oxidative stress. Recently, it was identified as a chaperone or as glutathione-independent glyoxalase. To unveil the role played by this protein in budding yeast cells, we investigated its involvement in the protection against diverse environmental stresses. Here, we show that HSP31 gene is controlled by multiple transcription factors, including Yap1p, Cad1p, Msn2p, Msn4p, Haa1p and Hsf1p. They mediate the HSP31 responses to oxidative, osmotic and thermal stresses, to toxic products of glycolysis: methylglyoxal and acetic acid, and to the diauxic shift. We also demonstrate that the absence of the HSP31 gene sensitizes cells to these stressors.

Overproduction of Hsp31p rescues the sensitivity of glo1Δ cells to methylglyoxal and the increased sensitivity of the ald6Δ strain to acetic acid. We postulate that *S. cerevisiae* Hsp31p may have broader substrate specificity than previously proposed and is able to eliminate various toxic products of glycolysis. Elucidating the role of this protein will bring us closer to unraveling the molecular function of its medically important orthologs, human DJ-1 and *C. albicans* Glx3 proteins.

[13]

VDAC and cell viability of the inducible PC12 model of Huntington's disease

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Huntington disease (HD) is a fatal neurodegenerative disorder characterized by a selective loss of neurons, especially from the striatum and deep layers of cerebral cortex. The disease belongs to polyglutamine expansion diseases because is caused by CAG trinucleotide repeat expansion in exon 1 of HTT gene encoding huntingtin (Htt). The repeats number higher than 35 results in its mutant form (mHtt) regarded as HD triggering factor. It is now obvious that mitochondria play a vital role in HD pathogenesis while Voltage-Dependent Anion selective Channel (VDAC) is described as crucial for the organelle functioning. Therefore we decided to estimate the effect of Htt and mHtt expression on VDAC functioning. For that purpose we applied HD model based on PC12 cells derived from a pheochromocytoma of the rat adrenal medulla. The model consists of PC-12HD-Q23 and PC-12HD-Q74 cells with induced (doxycycline) and monitored (GFP labeling) expression of Htt and mHtt, respectively. The obtained results including functional properties of VDAC isolated from PC-12HD-Q23 and PC-12HD-Q74 cells indicate that VDAC may constitute an important element of cytotoxic effect caused by mHtt.

The PC12 cell lines were obtained from David Rubinsztein and Andreas Wyttenbach, UK.

[14]

ATM gene alterations in Polish patients with ataxia-telangiectasia

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Ataxia-telangiectasia (AT, MIM#208900) is a complex genetic neurodegenerative and immunodeficiency disorder, is inherited in an autosomal recessive manner. AT is characterized by cerebellar degeneration, immunodeficiency, premature aging, cancer predisposition, and radiation sensitivity. AT results from mutations in the ataxia telangiectasia mutated gene (ATM).

We screened 105 samples from 49 AT families using cDNA sequencing and multiplex ligation-dependent probe amplification.

58 ATM mutations were identified in 40 patients (72.5%), among which 7 mutations have not previously been reported. New detected variants are: c.8441delC, c.6145T>G, c.434T>G, c.6754_6754delAfsX5, c.4007_4008insA, c.7606G>A and simultaneous deletion of 62 and 63 exons of gene. The mutation types are diverse, including nonsense (47.5% all detected mutations), splicing (38.9%), missense alterations (15.3%) and 1 large genomic deletion. Only 2 mutations have been found in homozygous state ([c.4007_4008insA];[c.4007_4008insA]), ([c.9021_9022insA]; [c.9021_9022insA]). Most frequent mutations among our AT patients are: c.5932G>T (7), c.6095G>A (10), c.7630-2A>C (11).

In this study, we confirmed status of recurrent mutations, but also detected new changes in ATM sequence. Most of the Polish patients are compound heterozygotes and none of them having the same combination of mutations, what makes molecular diagnostic more difficult.

[15]

Gender differences in cell death in cells harboring Leber's hereditary optic neuropathy mutations

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Leber's hereditary optic neuropathy (LHON) is a maternally inherited form of central vision loss due to optic nerve degeneration caused by point mutations in mitochondrial DNA (mtDNA). In most cases, the mutated mtDNA is present at about 100% in every cell, but only retinal ganglion cells (RGC) are affected. LHON in general has an early onset between the age of 20 and 30 years and male preponderance (men are four to five times more likely to develop the disease). The aim of this study is to determine gender differences in cell death under the influence of sex hormones.

Primary results have shown no difference in the level of apoptosis in LHON affected males and healthy controls after application of both testosterone and estradiol. The obtained results confirmed findings of other authors that cell death in cells with LHON mutations takes place via a caspase-independent pathway. In the female control cell line the level of apoptosis was much higher after induction of oxidative stress and application of testosterone even in low concentrations, but in regular conditions the same levels of testosterone did not trigger apoptosis.

[16]

AMPA receptors modulate Store-Operated Calcium Entry and interact with STIMs in rat cortical neurons

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The process of store-operated calcium entry (SOCE) leads to refilling the 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-located Ca^{2+} channel protein (ORAI1) mediates the formation of complexes and underlies SOCE in non-excitable cells. Our previous data indicated that STIMs are involved in Ca^{2+} homeostasis in neurons, form complexes with endogenous ORAI1, but play a distinct role in SOCE. 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 endogenous STIM1 and STIM2 interact with endogenous GluA1 and GluA2, AMPA receptors (AMPARs) subunits, using co-immunoprecipitation assays. To assess the role of AMPARs in SOCE, they were inactivated by their specific inhibitors. Single-cell Ca^{2+} measurements showed that in the presence of NBQX or CNQX SOCE was ~3.7 or ~2.2 times decreased, respectively. In addition, AMPA-induced calcium signal was reduced by 80% or by 53% by SOCE inhibitors, ML-9 or SKF96365, respectively. Altogether, our data suggest that STIMs in neurons can control AMPA-induced Ca^{2+} entry as a part of the mechanism of SOCE.

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

[17]

Electrochemical Biosensors for Detection of Alzheimer's Disease Markers

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Alzheimer disease is the most common form of dementia. Therefore the development of biosensors destined for screening of naturally occurring compounds, which might be used as the preventing agents, as well as for early diagnostics of Alzheimer disease is very demanding. We proposed the several biosensors destined for the above purposes.

The immobilization of Aβ1-40 was performed on Au-colloid modified gold electrodes as well as on the HS-aliphatic acid monolayer through EDC/HNS activation. Theses types of biosensors were used for determination of interaction between Aβ1-40 and selected alkaloids.

In the next type of biosensors, RAGE domains were covalently immobilized on the redoxactive monolayer

through interactions with polyhistidine tag. These biosensor were applied for determination Aβ peptides, S100B protein and glycated albumin, the potential markers of Alzheimer disease.

Cyclic voltammetry and electrochemical impedance spectroscopy and surface plasmon resonance were used as the measuring systems. The presented biosensors might be very useful tools for early diagnosis of Alzheimer disease.

This research was supported by grant no: POIG.01.01.02-00-048/09.

[18]

Dissociation of amyloid aggregates with photo-switchable molecular levers

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As a consequence of the society aging in Europe, the occurrence of cognitive impairment and dementia is rapidly becoming a significant challenge. Up to 70% of dementia cases in EU is due to the Alzheimer's disease (AD) – a neurodegenerative disease with no cure. With the increasing proportion of the elderly among Europeans, this problem is dramatically growing, especially in Western Europe, where the population suffering from dementia is reaching now ~7.5 million people. Moreover, AD is becoming a severe economic issue. The cost of dementia for the 2015 in Europe has been estimated for 200 billion Euros, and increased by 25% in the last five years. Despite huge and long term research efforts there is still no cure for AD. Considering that, a question rises – where else shall we look for new and effective treatments? We believe that the answer for this big question may lie in a newly designed derivatives of a small but very portentous photoresponsive molecule called azobenzene.

[19]

Two faces of one disease – difference in cell cycle regulation and apoptotic response between lymphocytes from familial and sporadic Alzheimer's disease patients

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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. A growing body of data has 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. Recently we have demonstrated that lymphocytes from the sporadic form of AD (SAD) show G1 phase arrest and increased levels of protein p21, the key regulator of apoptosis and the G1/S cell cycle checkpoint. Since it is known that p21, besides controlling the G1/S checkpoint, can regulate apoptosis, we conducted studies to determine if 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) aremore resistant to 2dRib-induced cell death than control or SAD cells. p21 mRNA and protein levels significantly increased in FAD cells in response to 2dRib. In addition, 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 a gain of function effect over wtPS1. Altogether, our results showed that the mechanism of apoptotic response to acute oxidative stress distinguishes cells from SAD and FAD patients.

[20]

Potent 5-HT6 receptor antagonists for the treatment of Alzheimer's disease

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Alzheimer's disease, an irreversible neurodegenerative disorder, constitutes one of the most frequent forms of dementia. AD is characterized by progressive deterioration of cognitive functions. In recent years 5-HT6 receptor has emerged as a promising molecular target for the treatment of cognitive deficits associated with AD [1].

Herein we present the development of novel class of 5-HT6R antagonists based on 1H-pyrrolo[3,2-c]quinoline core. The study allowed for identification of compound 14 (S)-1-[(3-chlorophenyl)sulfonyl]-4-(pyrrolidine-3-yl-amino)-1H-pyrrolo[3,2-c]quinoline, more selective and potent 5-HT6R antagonist than the reference compound SB-742457. Further evaluation of 5-HT6Rs constitutive activity at Gs signaling revealed that 14 behaved as a neutral antagonist, while SB-742457 was classified as an inverse agonist [2,3].

Compounds 14 and SB-742457 reversed phencyclidine memory deficits and displayed procognitive properties in cognitively unimpaired animals in NOR tasks. Additionally, compound 14 demonstrated higher anxiolytic effect than SB-742457 in Vogel test and showed similar antidepressant-like properties in FST.

These results support therapeutic potential of 5-HT6R antagonists and inverse agonists in the treatment of cognitive decline associated with neuropsychiatric disorders like autism, Alzheimer's or Parkinson's disease.

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[21]

New rare variants of TREM 2 gene involved in neurodegeneration

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Introduction: The genetic basis of late-onset Alzheimer disease is complex. Variation in multiple genes have been suspected to lead to the disease. Several candidate genes are involved in immune response and inflammation pathways. TREM2 (triggering receptor expressed on myeloid cell2) is one of them. It is known to be expressed on the cell membrane of a subset of myeloid cells, including microglia. TREM2 increases phagocytic pathways and suppresses inflammatory reactivity. Reduce function of TREM2 may be connected with Alzheimer's conditions and other neurodegenerative disorders. Homozygous loss-of-function mutations in this gene cause Nasu-Hakola disease. Several studies have reported the TREM2 R47H rare variant to be a risk factor for AD.

Methods: TREM2 exon2 in 208 neurologically normal controls, 274 AD, 194 ALS and 135 FTD patients were sequenced.

Results: Nine rare variants located in exon 2 of TREM2 were identified. Seven of them were reported previously. Novel synonymous variant (G29G) and single nucleotide insertion in 3' intron splice site (c.41-2_3insA) were identified for the first time, only in ALS patients.

Conclusion: Little is known about the biochemical mechanisms that underline the connection between neurodegeneration and TREM2 however the results indicate that rare variants are associated with an increase in neurodegenerations susceptibility.

[22]

Combined metabolomics and transcriptomics approaches to assess the IL-6 blockade as a therapeutic of ALS: deleterious alteration of lipid metabolism

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Playing an important role in the regulation of systemic metabolic regulation and neuroinflammation, interleukin-6, a major cytokine of the inflammatory response, has been proposed as a target for management of amyotrophic lateral sclerosis. Although one pilot clinical trial provided promising results in humans [1], another one recent preclinical study showed that knocking-out interleukin-6 gene in mice carrying amyotrophic lateral sclerosis did not improve clinical outcome [2]. We aimed to determine the relevance of the IL-6 pathway blockade in a mouse model of ALS, by using a pharmacological antagonist of interleukin 6, a murine surrogate of tocilizumab, namely MR16-1. We first characterized immunological and metabolic status of untreated SOD1*G93A (mSOD1) mice comparatively to wild-type mice, and then we compared treated versus untreated mSOD1 mice.

Metabolomics and transcriptomics analyses revealed that metabolic effects of IL-6 blockade were mild compared to metabolic disturbances observed in ALS condition, which include especially tryptophan, arginine and proline metabolism pathways (including polyamines). MR16-1treatment mainly affected lipid metabolism. Immunological analysis showed a significant increase of regulatory T cells count (p = 0.0268) and a decrease of CXCL1 (mKC) concentrations in plasma (p = 0.0479). Finally, a deleterious clinical effect of MR16-1 was revealed, with a speeding up onset of weight loss (p = 0.0041) and decreasing body weight (p < 0.05).

As metabolic pathways involved in MR16-1 therapy have been previously clearly described in ALS, we may suspect that IL-6 blockade had negative effect through a multiparametric effect, despite a significant anti-inflammatory effect. Together, these results indicate that IL-6 blockade did not improve clinical outcome of mSOD1 mouse model of ALS.

Key words: interleukin-6, metabolomics, transcriptomics.

ARSLA, Fondation Brou de Laurières, and INSERM

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DOI: 10.5114/fn.2016.64825

Appendix to the abstracts of the 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

Abstracts from poster session was printed in "Folia Neuropathologica" 3/2016

Lectures

Thursday, November, 17th, 2016

Methamphetamine-induced aberrant neurogenesis in the hippocampus

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Disruption of the blood-brain barrier (BBB) and the development a neurocognitive deficits have been identified as the primary events in methamphetamine (METH) abuse. It is now recognized that adult brains have progenitor cells, which differentiate into specific lineages, including neurons. A frequently overlooked fact is that these cells are in close proximity to the BBB. We hypothesize that METH-induced disruption of BBB impairs differentiation of neural progenitor cells to mature neurons, affecting neurogenesis. Physical exercise is known to promote cell survival and functional recovery after brain injuries. We also reported that preceding voluntary exercise protected against METH-induced disruption of the BBB (Toborek *et al*., Mol Neurodegener, 2013). Therefore, we assessed the impact of exercise, in a form of voluntary wheel running, on METH-induced abnormal neural differentiation.

While no effective therapy is available for the treatment of METH-induced neurotoxicity, behavioral interventions, including aerobic exercise, are being used to improve depressive symptoms and substance abuse outcomes. The present study focuses on the effect of exercise on METH-induced neurotoxicity in the hippocampal dentate gyrus (DG) in the context of the BBB pathology. Mice were administered with METH or saline (vehicle) by i.p. injections three times per day for 5 days with an escalating dose regimen in 4 h intervals, starting from 0.2 mg/kg. One set of mice was sacrificed 24 h post last injection of METH, and the remaining animals were either subjected to voluntary wheel running (exercised mice) or remained in sedentary housing (the sedentary group). METH administration resulted in decreased expression of tight junction (TJ) proteins and increased BBB permeability in the hippocampus. These changes were preserved post METH administration in sedentary mice and were associated with the development of significant aberrations of neural differentiation. Exercise protected against these effects by enhancing the protein expression of TJ proteins, stabilizing the BBB integrity, and enhancing differentiation of progenitor cells to neuronal lineage. In addition, exercise protected against METH-induced systemic increase in inflammatory cytokine levels. These results indicate for the first time that exercise protects against chronic METH-induced impaired hippocampal neurogenesis by enhancing BBB integrity and decreasing systemic production of proinflammatory cytokines, such as IL-1β and TNF-α.

DA039576, DA027569, HL126559, MH098891, MH072567, the Miami CFAR funded by MH063022, and by NSC 2015/17/B/NZ7/02985.

Micro and macro-scale approach of stem cell based developmental neurotoxicity testing

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Developmental neurotoxicity (DNT) in humans refers to the damage of the central nervous system (CNS) as the effect of exposure to an adverse substance during *in utero* and early postnatal development. It is widely accepted, that developing brain in children and fetuses is much more vulnerable to chemical perturbation than the adult brain. DNT often lead to the loss of cognitive skills, autism, ADHD and, what's more, may also cause silent damage manifesting itself after number of years by contributing to neurodegenerative diseases such as Parkinson's or Alzheimer's diseases. Developing reliable human based *in vitro* systems to study drug toxicity and their mode of action is a major challenge for establishing new and safe DNT therapies. However, research efforts are hampered by limited access to human tissue, especially those that represent the earliest stages of development of human embryos.

Both ethical and technical concerns regarding the use of human embryo or fetuses for derivationof neural stem cells can be circumvent by the possibility to acquire human induced pluripotent stem cells (hiPSC) that are similar to human embryonic stem cells (hESC), but can be generated from any adult tissue in the body. For this reason in our studies we have applied innovative, patient-specific approach to obtain human induced pluripotent stem cells (hiPSC) – based 3D culture of organoid spheres called "embryonic bodies" (EB), that recapitulate the earliest stages of development and can be successfully committed for neural differentiation. The composition of the obtained 3D aggregates/spheres included cells expressing typical markers of the three germ layers, which in proper differentiating conditions can acquire advanced neuronal

markers MAP-2, Doublecortin, Synapsin, TH as well as glial markers: PDGFRa, GFAP and GalC. ThehiPSC-derived EBs cultures at different stages of differentiation were investigated by our group for MeHgCl induced embryotoxicity and genotoxicity.

The advancement to the DNT screening possibilities in stem cell-based culture systems is provided by the emerging technologies, which are implicated to create high content/high throughput drug discovery *in vitro* platforms that are useful in filling the gap between animal testing and clinical trials. There are two different strategies to create such advanced research "biomimetic" *in vitro* systems. The first is to establish "microscale" environments to test cell behavior and molecular mechanisms, even at the single cell resolution. The second approach is to provide a "macroscale" structural, biomaterial based 3D template for cell differentiation and function, that allows the growth of complicated human tissues and organoids. The ability to use conditional bioengineering to manipulate biomaterials in "real time", is emerging as a powerful tool in regulating behavior of stem cells that are encapsulated in the scaffolds. Both, micro- and macroscale systems provide new tools to screen *in vitro* for chemical effects on the critical DNT events, such as proliferation, migration, neurite outgrowth or synaptogenesis. In the previous studies of our group the micro-scale engineering techniques (surface patterning: micro-contact printing and piezzoelectric spotting) were used to control cell microenvironment interactions (cell-cell, cell-ECM, and cell-soluble factor interactions) as well as cellular processes (proliferation, migration, differentiation) in the culture of human neural stem cells, that were immobilized to the bioactive surface and exposed to developmental neurotoxicant (e.g. MeHgCl).

This talk will provide the state of the art on the development of human stem cell based *in vitro* systems for DNT testing, with stem cell 3D models and micro/nano engineered drug screening platforms, used to test variety of compounds. The results of our group implementing both: "micro" and "macro"- scale approach to DNT testing will be discussed in the context of the advancement in the field.

Supported by the statutory funds to MMRC and NSC Grant 2013/11/B/NZ1/00089.

Endocrine disruptors and their neurotoxic effects on developing brain

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Endocrine disrupting chemicals (EDCs) are environmental organic compounds which are able to interfere with hormone receptors, hormone synthesis or hormone conversion, thus altering the hormone-dependent processes and disrupting functions of endocrine glands. In addition to EDC properties, organic compounds such as dioxins, polychlorinated biphenyls (PCBs), pesticides (e.g. DDT, DDE), brominated flame retardants, plasticizers (e.g. nonylphenol) and personal care products possess capacities of altering neural transmission and neural networks. Although EDCs are known to cross blood-brain barrier, little is known about their impact on the nervous system, especially at early developmental stages. Systematic and complex data concerning mechanisms of actions of EDCs in neuronal cells are missing. Recognition of these mechanisms is particularly important because EDCs through alteration of epigenetic status and dysregulation of apoptosis and autophagy could impair neural development and/or cause neurotoxicity and neurodegenerations. Furthermore, interactions of EDCs with steroid and xenobiotic receptor signaling pathways at early stages of neural development could cause abnormalities which might reveal in adolescent or adult nervous system. Interestingly, epidemiological data showed correlations between exposures to environmental pollutants and increased risk of neuropsychiatric disorders, including autism, attention deficit and hyperactivity disorder, learning disabilities, aggressiveness and depression. Exposalto pesticides or PCBs has been associated with neural degenerations, involving Parkinson's and Alzheimer's diseases. Recently, we have demonstrated that stimulation of aryl hydrocarbon receptor (AhR)-signaling and impairmentof G-protein coupled receptor 30 (GPR30)-signaling play important roles in the propagation of DDT-induced apoptosis in mouse neurons. We have also shown that the stimulation of retinoid X receptor (RXR)-mediated signaling is important for DDE-induced apoptosis and neurotoxicity that is accompanied by global DNA hypomethylation. Moreover, we provided evidence on key involvement of RXR/pregnane X receptor (PXR)/constitutive androstane receptor (CAR) signaling pathways in the apoptotic and neurotoxic actions of nonylphenol. These new data give prospects for understanding the neurodevelopmental pathomechanisms of actions of EDCs. Targeting xenobiotic nuclear receptors could be asset in searching for effective neuroprotective

strategies against EDCs and their controlled use, especially during the early stages of neural development.

This work was supported by the Operating Program of Innovative Economy 2007-2013, grant No. POIG.01.01.02-12-004/09 and the statutory fund of the Institute of Pharmacology Polish Academy of Sciences, Cracow, Poland.

Triggering role of Ca^{2+} imbalance in the induction of acute oxidative stress and cytotoxicity in primary cultures of rat cerebellar granule cells challenged with TBBPA

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The aim of this study, performed using primary cultures of rat cerebellar granule cells (CGC), was to determine the role of increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in inducing oxidative stress and cytotoxicity in neurons acutely treated with brominated flame retardant tetrabromobisphenol A (TBBPA). Neuronal cultures were exposed for 30 minutes to 10 or 25 μM TBBPA. Changes in $[Ca^{2+}]_i$, in the production of reactive oxygen species (ROS), and in the potential of mitochondria ($\Delta\Psi m$) were measured fluorometrically in CGC during exposure to TBBPA, the intracellular level of glutathione (GSH) and catalase activity were determined immediately after incubation, and cell viability was evaluated 24 h later. Application of TBBPA concentration-dependently increased $[Ca^{2+}]_i$ and ROS production; it reduced also GSH content, catalase activity, $\Delta\Psi m$ and neuronal viability. Antagonists of NMDA and ryanodine receptors which, when applied in combination, completely inhibited rises in $[Ca^{2+}]_i$ evoked by both concentrations of TBBPA, only partially reduced neuronal death. They entirely prevented oxidative stress and drop in $\Delta\Psi m$ induced by 10 μM TBBPA, while these effects of 25 μM TBBPA were only partially reduced. Cyclosporin A did not prevent TBBPA-evoked drop in $\Delta\Psi m$ and ROS production, but was partially cytoprotective, exclusively at high concentrations against toxicity of 10 μM TBBPA. Free radical scavengers significantly reduced indices of oxidative stress in CGC treated with TBBPA and improved their viability, but did not interfere with rises in $[Ca^{2+}]_i$ and drop in $\Delta\Psi m$, while their co-administration with NMDA and ryanodine receptor antagonists almost completely protected the cells. In conclusion, both, Ca^{2+} imbalance and oxidative stress mediate acute toxicity of TBBPA in CGC. TBBPA-induced increase in $[Ca^{2+}]_i$ is a primary and major event which triggers oxidative stress and depolarization of mitochondria in CGC. At high TBBPA concentration Ca^{2+}-independent portion of oxidative stress and cytotoxicity was revealed.

This study was supported by the Polish National Science Centr, grant no. 2012/05/B/NZ7/03225.

Glutaminase, HIV-associated neurocognitive disorders and beyond

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Glutamate serves as a crucial excitatory neurotransmitter that is essential for the proper functioning of the brain. However, the excess level of glutamate proves to be neurotoxic and contributes to the pathogenesis of neurodegenerative dissease, indlucing HIV-1 associated neurocognitive disorders (HAND). HIV-1-infected and/or immune-activated microglia and macrophages are pivotal in the pathogenesis of HAND. Glutaminase, a metabolic enzyme that facilitates glutamate generation, is upregulated and may play a pathogenic role in HAND. Our previous studies have demonstrated that glutaminase is released to the extracellular fluid during HIV-1 infection and neuroinflammation. However, key molecular mechanisms that regulate glutaminase release remain unknown. Recent advances in understanding intercellular trafficking have identified microvesicles (MVs) as a novel means of shedding cellular contents. We posit that during HIV-1 infection and immune activation, microvesicles may mediate glutaminase release, generating excessive and neurotoxic levels of glutamate. MVs isolated through differential centrifugation from cell-free supernatants of monocyte-derived macrophages (MDM) and BV2 microglia cell lines were first confirmed in electron microscopy and immunoblotting. As expected, we found elevated number of MVs, glutaminase immunoreactivities, as well as glutaminase enzyme activity in the supernatants of HIV-1 infected MDM and lipopolysaccharide (LPS)-activated microglia when compared with controls. The elevated glutaminase was blocked by GW4869, a neutral sphingomyelinase inhibitor known to inhibit MVs release, suggesting a critical role of MVs in mediating glutaminase release. More importantly, MVs from HIV-1-infected MDM and LPS-activated microglia induced significant neuronal injury in rat cortical neuron cultures. The MV neurotoxicity was blocked by a glutaminase inhibitor or GW4869, suggesting that the neurotoxic potential of HIV-1-infected MDM and LPS-activated microglia is dependent on the

glutaminase-containing MVs. These findings support MVs as a potential pathway/mechanism of excessive glutamate generation and neurotoxicity in HAND and therefore MVs may serve as a novel therapeutic target.

Glutaminase, ammonia and hepatic encephalopathy: an opportunity for therapy

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Hepatic encephalopathy (HE) is a major complication of liver cirrhosis, and is classified into three types: Type A (acute) HE is due to with acute liver failure (ALF); Type B (by-pass) HE is due to portal-systemic shunting without intrinsic liver disease; and Type C (cirrhosis) HE occurs in patients with underlying cirrhosis. However, the appearance of hepatic encephalopathy in patients with acute-on-chronic liver failure was not included in this classification. HE manifests as a spectrum ranging from minimal disturbances in mental function that impacts on attention, cognition and quality of life to coma. Hepatic encephalopathy is a complex neuropsychiatric syndrome in patients with liver dysfunction or porto-systemic shunts. Stages of HE have been defined by West-Haven criteria: Stage 0 means no abnormality detected. Stage 1 trivial lack of awareness with shortened attention span, euphoria and anxiety and inability to do easy calculations. Stage 2 is characterized by lethargy, disorientation for time, changes in personality, inappropriate behaviour. Stage 3 was defined by somnolence and semi stupor, keeping response to stimuli with confusion, gross disorientation for time and space and bizarre behaviour. Stage 4 was defined by coma.

HE in patients with cirrhosis decompensation without criteria for ACLF has been strongly related to previous episodes of hepatic encephalopathy and the abuse of diuretics, but not with hyponatremia, infections or alcohol binge. Interestingly, GI bleeding seems to protect against HE instead to promote it. Improvement in the management of variceal bleeding avoiding infections and controlling bleeding could explain, at least in part, this result. On the other hand, in patients with ACLF, HE was also associated with previous bouts of overt HE but not with diuretics abuse, GI bleeding, alcohol binge or infections. These precipitant factors were equally distributed in patients with and without HE. The strong association between previous bouts of overt HE and HE support the hypothesis of the impact of gene alteration on the risk of developing HE. A microsatellite in the promoter region of gutaminase type K gene has been associated with increased risk of HE (form long-long of the microsatellite). However, other genes could be implicated on HE and a GWAS analysis is warranted to define the genetic profile associated with risk of overt HE in cirrhotics. Diuretics-induced renal insufficiency seems to be a major cause of HE in cirrhotics with acute decompensation, highlighting the role of kidneys on HE. Brain impairment appeared as consequence of hyperammonemia in the brain, oxidative stress, activation of microglia, hyponatremia and benzodiazepine-like substances able to promote an astrocyte-neuron dysfunction, neurological basis for HE.

In the management of patients with HE and liver dysfunction is mandatory to exclude other causes of neurological or psychiatric disorders and keep in mind other types of encephalopathy like sepsis or hyponatremia. Mental status should be explored using Glasgow scale. Nutritional assessment should also be included. Biochemical analysis include: full blood count, liver and kidney function, electrolytes, ammonia, thyroid function, inflammatory reactant, glycaemia, vitamin B_{12} and urine analysis. Patients with HE and ACLF should be admitted in the intensive care unit. The first step is removing any precipitant factor or treating it (infections by antibiotics; diuretics abuse: volume expansion; alcohol binge: thiamine and in cases of malnutrition nutritional support). If no precipitant factor was detected with have to focus on modulation of inflammation plus ammonia lowering drugs. In patients without response and preserved liver function, large porto-systemic shunts should be ruled out and embolised if present. Lastly, liver transplantation remained as the therapeutic option in patients with HE without response to all mentioned measures.

Several ammonia-lowering drugs are also able to avoid glutamine accumulation (that could serve as substrate for glutaminase transforming it into glutamate and ammonia – Trojan Horse hypothesis) excreting by urine it in form of phenylacetyl-glutamine. Ornithine-phenylacetate and glycerol or sodium phenylacetate belonged to this type of drugs. CB-839 a glutaminase inhibitor demonstrated in portacaval shunted rats its ability as ammonia lowering drug. The role of these drugs in management of overt HE requires future studies.

Conflict of interest

Manuel Romero-Gómez was inventor of THDP-17, a glutaminase inhibitor, which was licensed by Janus Development, S.L. He has ongoing research collaboration with Umecrine, S.A., Sweden. He has also received speaker fees from Bama-Geve, Merz and Norgine, S.A.

Brain glutaminase in psychiatric disease: from mouse models to schizophrenia

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Glutaminase (GLS1) is the rate-limiting step in the recycling of neuronal glutamate. GLS1 gene expression is highest in the hippocampus and cortex in wild-type mice. Mice heterozygous for a null-STOP mutation in GLS1 ($GLS1^{+/-}$ mice) display reduced GLS1 expression in hippocampus and prefrontal cortex, and reduced glutamate levels in these regions. Furthermore, fMRI imaging of $GLS1^{+/-}$ mice points to a focal reduction in hippocampal activity, mainly in the CA1 subfield. This finding contrasts with recent studies that demonstrate CA1 hyperactivity in patients with schizophrenia, prodromal patients and mouse models of increased glutamate transmission. $GLS1^{+/-}$ mice also demonstrate an attenuated behavioral and neurochemical response to the psychotomimetic drug amphetamine, and behavioral alterations in hippocampus-dependent and independent tasks. Interestingly, changes in NMDA receptor gene expression patterns in $GLS1^{+/-}$ mice emerge in adulthood but not in adolescence. Taken together, these findings support the centrality of GLS1 to normal hippocampal function, and indicate that GLS1 inhibition in adulthood may be a therapeutic target in treating schizophrenia-related abnormalities.

Glutamine addiction in brain cancer

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Cancer cells develop and succeed by shifting to different metabolic programs compared with their normal cell counterparts. One of the classical hallmarks of cancer cells is their high glycolytic fluxes even in the presence of abundant O_2 and heightened levels of lactate produced (Warburg effect). Another common metabolic feature of cancer cells is a high rate of glutamine (Gln) consumption normally exceeding their biosynthetic and energetic needs. The term Gln addiction is now widely used to reflect the strong dependence shown by most cancer cells for this essential nitrogen substrate after metabolic reprogramming. A Gln/Glu cycle occurs between host tissues and the tumor in order to maximize its growth and proliferation rates. Support for the existence of this cycle *in vivo* has come also from studies on enzymatic activities of glutamine synthetase and glutaminase in host tissues during tumor development. In this presentation, we review glutaminolysis in tumor cells by focusing on glutaminase proteins and with special emphasis on brain cancer. The mechanistic basis for this altered metabolic phenotype and how these changes are connected to oncogenic and tumor suppressor pathways are becoming increasingly understood. Based on these advances, new avenues of research have been initiated to find novel therapeutic targets and to explore strategies that interfere with glutamine metabolism as anticancer therapies.

Friday, November, 18th, 2016

Engineered human neural stem cells for treating spinal cord gliomas: a neurobiology-based approach

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There are currently no experimental models showing autonomic dysfunction for intramedullary spinal cord gliomas (ISCG), a lethal disease with no effective treatment. We have developed a rat model of ISCG and determined whether genetically engineered human neural stem cells (hNSC) could be developed into potent therapies for ISCG. ISCG rats received injection of hNSC.CD-TK, hNSC.CD or hNSC.CD-TK debris adjacent to the tumor epicenter 7 days after glioma cell implantation, followed with daily prodrug administration (5-FC and GCV; i.p. throughout the study). Post-tumor survival was assessed by time lasted before loss of body weight-bearing stepping in the hindlimb. Also evaluated were autonomic functions and tumor growth rate *in vivo*. ISCG rats with hNSC.CD-TK treatment showed significantly improved survival than controls that received hNSC.CD or hNSC.CD-TK debris ($P < 0.05$, median rank test), with better maintained autonomic function and reduced tumor growth rate. hNSC.CD-TK cells migrated diffusively into ISCG clusters to mediate targeted oncolytic effect in manners that spared spinal cord projection pathways. Through impeding glioma growth and preserving spinal cord neurobiology, dual gene-engineered hNSC regimen significantly prolonged survival in

a rat model that emulated sensorimotor and autonomic dysfunctions of human cervical ISCG. Our findings may provide a stem cell-based multimodal approach to treating ISCG and help formulate a recovery neurobiology-based therapeutic strategy for gliomas.

Hematopoietic stem cell-based therapy in neurodegenerative disorders – cellular and humoral mechanisms

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Advance of research on pathophysiology of stem cells (SCs) gives a chance on elucidation of the mechanisms regulating both normal human development as well as pathologic processes. Recently, a growing body of evidence suggests that SCs might be used in the adjuvant therapy of severe neurodegenerative disorders. Although there is currently a lack of compelling evidence for the effectiveness of cell-replacement therapies, the main benefit that is accessible from transplanted cells would be paracrine secretory activity, which is theoretically more convincing and fits nicely into the concept of adjuvant/supportive roles for SC-based therapy. Neurotrophic factors regulate survival, development, and function of nervous tissue. Illumination of their physiological role in the maintenance of central nervous system homeostasis as well as regeneration of damaged tissue have ignited expectations to heal neurodegenerative diseases, including amyotrophic lateral sclerosis. It has been demonstrated that SCs that are genetically modified with viral vectors (e.g., MSCs transduced to express NT-4) are capable of long-term survival after transplantation when NTs (e.g., NT-4 or BDNF) are continuously delivered and that this survival results in significant improvements in functional parameters that are observed with objective methods. On the other hand, there are evidences for the presence of neurotrophins and their receptors in distinct hematopoietic cell populations, showing that these cells express NTs and NT receptors at both the mRNA and protein levels. Of note, NT expression is greater under stress-related conditions. Furthermore, bone marrow-derived Lin- SCs administeredvia a lumbar puncturenoticeable modulated expression of both NFs as well as angiopoetic and proinflammatory factors in the cerebrospinal fluid. Overall, the advances in experimental studies suggest that SC-based therapy might represent a novel treatment modality for the repair and regeneration of injured neural tissue. However, further extensive studies are definitely required to understand the mechanisms of SC actions, particularly their paracrine activities, and to present SCs as a new treatment option for clinical approaches.

Stem cell therapy for Parkinson's disease

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Studies in animal models of Parkinson's disease (PD) have shown that transplanted dopamine neuroblasts can restore dopaminergic neurotransmission in the grafted striatum and reverse PD-like motor impairments. Open-label clinical trials in patients with PD have shown that dopamine neuroblasts obtained from fetal human midbrain tissue can survive and function over many years in the brain of PD patients, restore striatal dopamine release, and provide sustained and long-lasting improvements in motor behavior. The ethical and practical problems associated with the use of fetal tissue isa serious obstacle to further developments of this approach. Further progress, therefore, is critically dependent on the development of transplantable dopamine neurons from stem cells. The most promising results so far have been obtained using pluripotent stem cells, ESCs or iPSCs, as starting material. Recently developed and optimized protocols allow efficient generation of midbrain dopamine neurons from human ES cells that survive well following transplantation to the striatum, in the absence of any contaminating tumor-forming cells, and differentiate into genuine midbrain dopamine neurons of both A9 and A10 subtypes. In recent experiments performed in immunosuppressed and immunodeficient rats we have shown that the hESC-derived neurons grow to form an extensive axonal terminal networks in appropriate striatal, limbic and and cortical targets and reverse PD-like motor impairments. The results indicate that transplantable and fully functional midbrain dopamine neurons can be generated from human ES cells, ready to be used in patients.

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New strategies of stem cell therapy in retinal disease

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In the retina the damage of retinal ganglion cells, retinal pigment epithelial cells and photoreceptors cells cause potentially blinding diseases as glaucoma, age-related macular degeneration, retinitis pigmentosa and others. Cell therapy has raised new hopes for the management of these diseases. *In vivo* studies and phase I-II clinical trials have shown promising results. However, several factors as cells source, route of administration, integration and function of cells into the retina as well as safety are still under close evaluation. New non-invasive techniques of molecular imaging of the retina might accelerate the process of clinical application of stem cells.

Challenging tasks and future perspectives of cell therapies in retinal diseases will also be discussed.

Stem cells for ALS: an overview of possible therapeutic approaches

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Over the past 25 years, stem cell technologies have become an increasingly attractive option to investigate and treat neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS). The pathogenesis of ALS remains unclear, multiple factors are thought to contribute to the progression of ALS, such as network interactions between genes, environmental exposure and impaired molecular pathways.

The neuroprotective properties of neural stem cells (NSCs) and the paracrine signaling of mesenchymal stem cells (MSCs) have been examined in multiple pre-clinical trials of ALS with promising results. The data from these initial trials indicate a reduction in the rate of disease progression. The mechanism through which stems cells achieve this reduction is of major interest. Here, we review up to-date pre-clinical and clinical therapeutic approaches employing stem cells, and discuss the most promising ones.

Stem cells and neurorepair – from bench to clinic and back

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Central nervous system is one of the least well-known structures in our body. This is due to its complicated structure, and the difficulties to study the system both *in vitro* and *in vivo*.

The consequence of brain damage is a motor disability of the patient as well as neurological degeneration. The cause of encephalopathy can be, among others, mechanical trauma, metabolic problems, infection or pregnancy poisoning. Current encephalopathy treatments e.g. pharmacotherapy and hypothermia followed by motor and neurological rehabilitation do not bring the expected results. This is at least partially due to a lack of understanding of the biological aspects of the regeneration of the damaged brain tissue and the possibility of intervention in that process, which could lead to improved health status of patients.

The results of our recent clinical study, conducted by a multidisciplinary team of translational researchers from Department of Transplantation UJCM and clinicians from Department of Neurosurgery UJCM demonstrated that autologous mesenchymal stem cells (MSC) transplantation in children with encephalopathy leads to an improvement of the clinical picture of patients, including increase motor skills and overall neurological recovery.

To determine the mechanism of MSC action on the central nervous system cells research has been conducted with the use of induced pluripotent stem cells differentiated into GABAergic neurons. Preliminary results indicate that factors MSC increased activity of GABAergic precursors and their neuronal differentiation potential.

The project was supported by the research grant from the National Science Centre UMO-2015/17/B/NZ5/00294.

Modulation of microglial activation by CD200R activation in models of neurodegeneration

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The main role of microglia, which are the primary immune cells of the brain, is protective, and it is generally considered that acutely-activated microglia, that retain their ability to return to the resting state, are responsible for protection. On the contrary chronic activation of microglia is probably the primary cause of the neuroinflammatory changes are a feature of many, if not all neurodegenerative diseases. Therefore a significant challenge is to understand the factors that drive microglial activation and to identify strategies that can manipulate microglial activation. It is known that interaction of microglia with other cells plays a part in maintaining microglia in a quiescent state and this is achieved by ligand-receptor interactions that result in neuroimmune modulation. One of these ligand-receptor pairs is CD200-CD200R. Here evidence will be presented which support the view that this interaction is important in neuroprotection in a number of neurodegenerative conditions and in endothelin-1-induced ischaemia. The possibility that this interaction can be exploited to optimize the beneficial effects of mesenchymal stem cells in a model of stroke will be considered.

Pre-transplantation optimization of MSC function for enhancing their regenerative potential

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Nowadays, when the regenerative medicine enters the clinic, there is a need for establishing precise protocols for stem/progenitor cell preparation. The results of published trials are diverse and depend on the source of stem cells, as well as the method of cell isolation and propagation.

To optimize MSC isolation and propagation techniques that could increase their neurogenic potential, neuroprotective properties, extend their survival and slow down their aging we have compared: (i) properties of freshly isolated vs neuraly commited MSC, (ii) cells isolated via mechanical vs enzymatic method and (iii) MSC cultured in normoxic vs physioxic* oxygen conditions.

Our experiments showed that the strongest ability for neuroprotection was provided by freshly isolated cells and the first cohort of migrating MSC cells (passage O). Along further passaging the cells phenotype changed substantially and cell neuroprotective effect declined together with modification of paracrine capabilities of WJ-MSC-secreted cytokines. These results will be challenged with our previous data gathered in preclinical and clinical experimentations showing that undifferentiated, SRTF** expressing MSC, capable to time-locked proliferation, migration and ultimately to neural differentiation are the most effective in various therapeutic transplantation models.

Our results show significant differences between MSC populations obtained with two methods (mechanical vs. enzymatic) of isolation. Despite comparable level expression of typical mesenchymal markers (CD73, CD90, CD105, CD166, Vimentin, Collagen, Fibronectin), mechanically isolated cells were more stable in culture, with shorter Population Doubling Time, higher ability to CFU-F formation and lower number of the senescent cells. Moreover a significantly higher expression of neural/neuronal markers: Nestin, β Tubulin III, GFAP, NF-200 and primitive marker α-SMA was observed. The preferable method seems to be the mechanical one. The method of cell isolation may substantially affect cell properties, determining their neural differentiation ability and presumably the neuroprotective properties. Therefore the efficiency cannot be the main determinant to choose the method of isolation.

We have focused also on the mechanism of adult type stem cells phenotypic plasticity evoked by culturing MSC in physioxic O_2 conditions. Results strongly suggest that induction of the less differentiated, SRTF-expressing, pluripotent-like state of MSCs significantly increase they proliferation, epigenetic stability, survival, and capability of cells to differentiation into neural as well as endothelial directions.

To guarantee the high quality of the obtained cells, we should go beyond the framework of criteria developed by ISCT and focus on a number of other extremely important features which could help to select this particular cell population.

**Normoxic conditions – 21% oxygen concentration; physioxic conditions – 5% oxygen concentration*

***SRTF – Stemness-Related-Transcription-Factors (Oct4A, Nanog, Rex1, Sox2)*

The work was supported by National Centre for Research and Development grant No STRATEGMED1/234261/2/NCBR/2014.

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