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Stimulation of endogenous neurogenesis as a novel therapeutic strategy in neonatal hypoxic-ischemic brain injury

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ABBREVIATIONS

Acetyl-H3 - *Acetylated Histone H3*

Akt - *PKB, Protein kinase B*

ANOVA - *Analysis Of Variance*

Arg-1 - *Arginase-1*

ATP - *Adenosine Triphosphate*

Bax - *B-cell lymphoma 2 associated X protein*

BBB - *Blood–Brain Barrier*

Bcl2 - *B-cell lymphoma 2*

BDNF - *Brain-Derived Neurotrophic Factor*

BLBP - *Brain Lipid-Binding Protein*

BrdU - *5-bromo-2'-deoxyuridine*

BSA - *Bovine Serum Albumin*

BVs - *Blood Vessels*

C - *Control*

CA3 - *Cornu Ammonis region 3*

CD271 - *$p75^{NTR}$, LNGFR (Low-affinity Nerve Growth Factor Receptor)*

CNS - *Central Nervous System*

contra - *contralateral*

CSF - *Cerebrospinal Fluid*

Cy3 - *Cyanine3*

DCX - *Neuronal migration protein doublecortin*

DG - *Dentate Gyrus*

DKO - *Double Knock-Out*

DNA - *Deoxyribonucleic Acid*

EAE - *Experimental Autoimmune Encephalomyelitis*

ECL - *Enhanced Chemiluminescence*

ED1/CD68 - *Cluster of Differentiation 68*

EDTA - *Ethylenediaminetetraacetic acid*

EGF - *Epidermal Growth Factor*

ELISA - *Enzyme-Linked Immunosorbent Assay*

ErbB4 - *Receptor tyrosine-protein kinase erbB-4*

ERK - *Extracellular signal-Regulated Kinase*

Fabp7 - *Fatty acid binding protein 7*

FITC - *Fluorescein isothiocyanate*

GABA - *γ-aminobutyric acid*

GDNF - *Glial cell line-Derived Neurotrophic Factor*

GFAP - *Glial Fibrillary Acidic Protein*

GPCR - *G Protein-Coupled Receptor*

HAT - *Histone Acetyltransferase*

HCl - *Hydrochloric acid*

HD - *Huntington's Disease*

HDAC - *Histone Deacetylase*

HDACi - *Histone Deacetylase Inhibitor*

HE - *Hematoxylin and Eosin*

HEPES - *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

HI - *Hypoxia-Ischemia*

HIE - *Hypoxic-Ischemic Encephalopathy*

HRP - *Horseradish Peroxidase*

HSP70 - *Heat Shock Protein 70*

Hu - *The histone-like HU (heat unstable) protein*

ICV - *Intracerebroventricular*

IGF-1 - *Insulin-like Growth Factor 1*

Ig-like - *Immunoglobulin-like*

IL - *Interleukin*

ipsi - *ipsilateral*

JNK - *c-Jun N-terminal Kinase*

KO - *Knock-Out*

LPS - *Lipopolysaccharide*

LSM - *Laser Scanning Microscope*

LTD - *Long-Term Depression*

LTP - *Long-Term Potentiation*

LV - *Lateral Ventricles*

Math1 - *ATOH1, Protein atonal homolog 1*

MCAO - *Middle Cerebral Artery Occlusion*

ML - *Molecular Layer*

mRNA - *messenger RNA*

Msi1 - *Musashi-1 , RNA-binding protein Musashi homolog 1*

MWM - *Morris Water Maze*

n/a - *not available*

Na_2HPO_4 - *Sodium phosphate dibasic*

NaCl - *Sodium chloride*

NAD - *Sodium dihydrogen phosphate*

NaH_2PO_4 - *Nicotinamide Adenine Dinucleotide*

NaOH - *Sodium hydroxide*

NeuN - *Neuronal Nuclei Antigen*

NeuroD - *Neurogenic Differentiation 1 transcription factor*

NFκB - *Nuclear Factor kappa-light-chain-enhancer of activated B cells*

NG2 - *Nerve/Glial antigen 2, Chondroitin sulfate proteoglycan 4*

NGF - *Nerve Growth Factor*

Ngn1 - *Neurogenin 1*

NMDA - *N-Methyl-D-Aspartate*

NO - *Nitric Oxide*

NPCs - *Neural Progenitor Cells*

NT - *Neurotrophin*

O4 - *late oligodendrocyte progenitor-specific marker*

OB - *Olfactory Bulb*

OF - *Open Field*

OPC - *Oligodendrocyte Progenitor Cell*

$P15^{INK4b}$ - *Cyclin-dependent kinase 4 inhibitor B*

p53 - *tumor suppressor protein 53*

Pax6 - *Paired box 6*

PBS - *Phosphate-Buffered Saline*

PFA - *Paraformaldehyde*

phospho-CREB - *phosphorylated cAMP (Cyclic Adenosine Monophosphate) Response Element-Binding protein*

PI3K - *Phosphoinositide 3-Kinase*

PLC - *Phospholipase C*

PMSF - *Phenylmethane Sulfonyl Fluoride, Phenylmethylsulfonyl Fluoride*

Prox1 - *Prospero homeobox protein 1 transcription factor*

PSA-NCAM - *Polysialylated-Neural Cell Adhesion Molecule*

qRT-PCR - *quantitative Reverse Transcriptase Real-Time Polymerase Chain Reaction*

Rac	*- Ras-Related C3 Botulinum Toxin Substrate*
Ras-MAP	*- Ras -Mitogen Activated Protein*
rCBF	*- Regional Cerebral Blood Flow*
rhEPO	*- recombinant human EPO (Erythropoietin)*
RIP2	*- Receptor Interacting Protein-2*
RIPA	*- Radioimmunoprecipitation assay buffer*
RMS	*- Rostral Migratory Stream*
RNA	*- Ribonucleic Acid*
RNAi	*- Ribonucleic Acid interference*
RT	*- Room Temperature*
S100β	*- S100 calcium-binding protein β*
SAHA	*- Suberoylanilide Hydroxamic Acid, Vorinostat*
SB, NaB	*- Sodium Butyrate*
SD	*- Standard Deviation*
SDS-PAGE	*- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis*
SEM	*- Standard Error of Measurement*
SGZ	*- Subgranular Zone*
siRNA	*- small interfering RNA*
SIRT	*- Sirtuin*
Sox2	*- SRY (sex determining region Y)-box 2*
SVZ	*- Subventricular Zone*
TBST	*- Tris-Buffered Saline with 0.1% Tween 20*
TEMED	*- Tetramethylethylenediamine*
TH	*- Tyrosine Hydroxylase*
Tris	*- Tris(hydroxymethyl)aminomethane*
Trk	*- Tropomyosin receptor kinase*
TSA	*- Trichostatin A*
TUC-4	*- TOAD [Turned On After Division]/ Ulip [Unc-33-like phosphoprotein]/CRMP [Unc-33-like phosphoprotein] -4*
USV	*- Ultrasonic Vocalizations*
VEGF	*- Vascular Endothelial Growth Factor*
VPA	*- Valproic Acid*
WB	*– Western Blot*

ABSTRACT

Neonatal hypoxic-ischemic encephalopathy (HIE) resulting from perinatal asphyxia is a main cause of morbidity and mortality in newborns and children. It affects 1-3 out of a 1000 full-term births, where 15-20% of infants die and 25% that survive develop neurological dysfunctions and disabilities such as cerebral palsy, epilepsy and spastic paresis. In spite of the decades of research which led us to a better knowledge of the pathological mechanisms of hypoxic-ischemic brain injury, the clinical use of potential neuroprotective drugs became avoided owing to their insufficiency and/or secondary undesirable side effects which may influence normal brain function. The discovery that neonatal hypoxia ischemia (HI) stimulates a neurogenic response led to the speculation that the central nervous system mounts intrinsic regenerative potential to repair itself due to persistent production of neural progenitor cells. Although neurogenesis after HI has been well documented, the capacity of endogenous regeneration seems to be rather limited and insufficient for replacing the lost neurons. Meanwhile, gathered evidence indicated that the treatment of adult animals with small compounds, such a histone deacetylase inhibitors (HDACis), administered after the onset of stroke, exert stimulation of brain neurogenesis by modulating targets of endogenous signaling pathways or epigenetic mechanisms. Despite ever-growing information concerning the effect of several HDACis in neurogenesis–associated processes in the experimental stroke model in adults, the proof of their relevance *in vivo* after neonatal HI injury is still missing.

Therefore, the aim of this work was to examine whether treatment with histone deacetylase inhibitor - sodium butyrate (SB) has neurogenic/neuroprotective effects in a rat model of neonatal hypoxia-ischemia.

Experiments were conducted on an established model of neonatal hypoxia-ischemia (HI) induced in 7-day old Wistar rats by a permanent unilateral common carotid artery ligation followed by systemic hypoxia (60 minutes in 7.6% O_2 in N_2). Pups were assigned to 4 experimental groups – (1) control group (vehicle treatment), (2) control animals (SB treatment), (3) animals which underwent HI (vehicle treatment), (4) animals which underwent HI (SB treatment). Rats subjected to HI or sham-operated were treated with subcutaneous injections of sodium butyrate (300 mg/kg body weight) or vehicle (saline) starting immediately after hypoxic exposure and lasting 5 consecutive days. Endogenous proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) cell incorporation. Animals were sacrificed at specific time points after brain injury (1, 2, 3, 6, 7, 9, 11, 14 and 28 days, depending on the type of analysis). The undamaged hypoxic hemisphere, as well as hemispheres obtained from age-matched sham-operated animals, served as controls. The brain damage was assessed by the weight deficit in the ipsilateral (HI injured) hemisphere relative to undamaged contralateral hemisphere. To define the cell phenotype immunohistochemical staining was

conducted with specific primary and secondary antibodies. The level of molecular mediators released by brain ischemia-injured cells that may be crucial for neurogenic response (such as transcription factors, neurotrophic factors, kinases ERK1/2 and AKT, HSP70, as well as pro- and anti-apoptotic proteins) were determined by immunochemical assays (ELISA, Fluorescence Assay, Western Blot). The levels of mRNA were estimated by qRT-PCR. The last stage of investigation involved monitoring animal behavior at the age of 33 to 83 (P33-83) to evaluate functional outcome.

The results of the present study showed that sodium butyrate treatment provides a protective/neurogenic effect in neonatal rats subjected to hypoxia-ischemia. The neuroprotective effect was associated with a reduction of brain infarct and inhibition of HI-induced inflammation. The neurogenic effects of SB were expressed by expanded population of neuroblasts and mature neurons in the subventricular zone (SVZ), expansion of oligodendrocyte precursor cells and increased level of neurotrophins. In contrast, the number of new mature granule cells did not reach the control level in the neurogenic subgranular zone of the hippocampus, which indicates a limited effect of SB on this stage of neurogenesis. In addition, SB did not appear to improve neurological outcome. It may be deduced that in conditions of the presented experiments the insufficient number of cells within the granular cell layer observed after inhibitor treatment does not lead to full compensation for the lost neuronal circuits.

STRESZCZENIE

Okołoporodowe stany niedotlenieniowo-niedokrwienne, jeśli nie prowadzą do zgonu noworodka, są przyczyną przewlekłych zaburzeń neurologicznych, do których między innymi należy mózgowe porażenie dziecięce, padaczka, zespoły opuszkowe i pozapiramidowe oraz niedowłady kurczowe. Pomimo znaczącego postępu wiedzy i lepszego rozumienia mechanizmów odpowiedzialnych za narastające w czasie zmiany patologiczne, podejmowane próby neuroprotekcji farmakologicznej nie przyniosły oczekiwanych efektów.

Możliwość regeneracji nieodwracalnie uszkodzonych komórek nerwowych na drodze naturalnej repopulacji pojawiła się z chwilą odkrycia zdolnych do neurogenezy komórek macierzystych w mózgu dorosłych osobników. Duże zainteresowanie wzbudziła obserwacja, że w wyniku niedokrwienia mózgu dojrzałego, ale także eksperymentalnej asfiksji okołoporodowej, endogenne komórki macierzyste mogą przejść w fazę przyśpieszonych podziałów i tworzyć komórki potomne, które migrują do miejsc uszkodzenia, gdzie stają się komórkami o dojrzałym fenotypie.

Aktywacja procesu neurogenezy w wyniku epizodu niedotlenieniowo-niedokrwiennego (hipoksyjno-ischemicznego) nie budzi wątpliwości, natomiast liczba nowopowstałych komórek neuronalnych nie jest wystarczająca, aby zastąpić neurony uszkodzone. Podejmowane próby interwencji farmakologicznej mające na celu pobudzenie procesów naprawczych tkanki nerwowej poprzez bezpośrednią modulację endogennych komórek macierzystych/progenitorowych wykazały, że inhibitory deacetylaz histonów (HDACi) stymulują powstawanie nowych neuronów w modelach niedokrwienia mózgu dorosłych zwierząt poprzez modulacje szlaków sygnałowych bądź zmiany epigenetyczne. Należy podkreślić, że nie podjęto dotychczas systematycznych kompleksowych badań nad wpływem inhibitorów HDACi na neurogenezę niedojrzałego mózgu po uszkodzeniu okołoporodowym. Nieliczne publikowane dane mają jedynie charakter fragmentaryczny i nie pozwalają na wyciągnięcie wniosków.

Dlatego też głównym celem prezentowanej rozprawy było sprawdzenie, czy inhibitor deacetylaz histonów – maślan sodu (SB), będzie miał działanie neurogenne/neuroprotekcyjne po hipoksyjno-ischemicznym uszkodzeniu niedojrzałego mózgu.

Badania przeprowadzono na siedmiodniowych oseskach szczurzych stada Wistar, obu płci, na ustalonym modelu hipoksyjno-ischemicznego (HI) uszkodzenia mózgu wg metody opisanej przez Rice i wsp. (1981). Zabieg polegał na jednostronnym podwiązaniu lewej tętnicy szyjnej wspólnej, a następnie poddaniu zwierząt 60 minutowej hipoksji (7.6% O_2 w N_2). Wyodrębniono cztery grupy doświadczalne: (1) zwierzęta kontrolne (sham), (2) zwierzęta kontrolne traktowane SB, (3) zwierzęta po HI, (4) zwierzęta po HI traktowane SB. Zwierzęta poddane HI uszkodzeniu oraz zwierzęta poddane

operacji pozorowanej (sham) otrzymywały podskórną iniekcję maślanu sodu (300 mg/kg) lub sól fizjologiczną bezpośrednio po hipoksji oraz przez 5 kolejnych dni. Dla określenia endogennej proliferacji zastosowano 5-bromo-2-deoksyurydynę (BrdU) (dootrzewnowo). Czas przeżycia zwierząt uzależniony był od typu przeprowadzanej analizy i wynosił 1, 2, 3, 6, 7, 9, 11, 14 i 28 dni od indukcji HI. Hipoksyjna półkula mózgu (kontralateralna, przeciwstawna) oraz mózgi zwierząt pozornie operowanych stanowiły kontrolę dla półkuli uszkodzonej w wyniku HI. Uszkodzenie mózgu określano ubytkiem wagi półkuli HI w odniesieniu do półkuli kontrolnej oraz barwieniem skrawków mózgu hematoksyliną-eozyną. Dla określenia fenotypów komórek zastosowano podwójne barwienia immunohistochemiczne wykorzystując specyficzne przeciwciała I- i II-rzędowe. Poziomy wybranych mediatorów uwalnianych w czasie ischemicznego uszkodzenia mózgu (czynniki transkrypcyjne, neurotrofiiny i ich receptory, kinazy ERK1/2 i AKT, HSP70, czynniki związane z apoptozą) określano metodami immunochemicznymi. Dla oznaczenia poziomu ekspresji mRNA zastosowano reakcję odwrotnej transkrypcji, a następnie Ilościową reakcję łańcuchowej polimerazy (qRT-PCR). Ostatnim etapem było przeprowadzenie wybranych testów behawioralnych: testu otwartego pola (Open field), testu Rotarod, pomiaru siły uchwytu (Grip test), Basen wodny Morrisa (Morris Water Maze), wokalizacji ultradźwiękowych (USV).

Uzyskane wyniki wskazują na neuroprotekcyjny/neurogenny wpływ podania maślanu sodu po neonatalnej hipoksji-ischemii indukowanej u 7-dniowych szczurów. Neuroprotekcyjny efekt SB wyrażony był zmniejszeniem uszkodzenia półkuli ipsilateralnej (hipoksyjno-ischemicznej) mózgu. Efekt neurogenny badanego inhibitora związany był ze wzrostem liczby neuroblastów oraz dojrzałych neuronów w strefie okołokomorowej (SVZ), progenitorów oligodendrocytów oraz poziomu czynników neurotroficznych, a także z osłabieniem odpowiedzi zapalnej. Niestety, podanie SB nie zwiększyło liczby nowych dojrzałych neuronów ziarnistych do poziomu kontroli w neurogennej strefie podziarnistej zakrętu zębatego hipokampa (SGZ). Nie zaobserwowano także istotnej poprawy funkcjonalnej zwierząt. Wyniki te wskazują, że maślan sodu w warunkach eksperymentalnych prowadzonych badań nie prowadzi jednak do całkowitej kompensacji i odbudowy sieci neuronalnej.

1. INTRODUCTION

1.1. NEONATAL HYPOXIA-ISCHEMIA

Perinatal hypoxic-ischemic (HI) brain injury contributing to neonatal encephalopathy is one of the most common causes of mortality and long term detrimental neurological disabilities such as cerebral palsy, epilepsy, cognitive, motor and sensory impairments, mental retardation or other neurophysiological and neuropsychological handicaps. Although there have been many improvements and advances in methodology as well as in knowledge about the pathophysiology of hypoxic-ischemic encephalopathy (HIE) it still remains a major health problem worldwide. It affects approximately 1 to 3 per 1000 live births in developed countries with even up to 26 per 1000 live births in the underdeveloped world. About 20-25% of term newborns die and around 25% of those that survive develop serious adverse neurological outcomes. The massive personal and economic burdens generated by long-term neurological morbidity, together with the high prevalence of perinatal brain damage, are of considerable concerns, particularly as no effective treatment is available to date (O'Shea, 2008).

1.1.1. Etiology

In general, neonatal encephalopathy resulting from HI is likely to be a multifactorial condition with complex etiology, encompassing several casual events.

In term newborns, hypoxic ischemic brain injury can occur during the antepartum, intrapartum, or postnatal period, however there are also preconceptional risk factors for asphyxia including maternal age over 35 years, social factors, family history of seizures or neurologic disease, infertility treatment, or previous neonatal death.

Various issues or medical complications may lead to HIE during pregnancy. These include: maternal diabetes with vascular disease, problems with blood delivery to the placenta, pre-eclampsia, cardiac disease, maternal prothrombotic disorders and pro-inflammatory states, drug and alcohol abuse, severe fetal anemia, lung malformations, intrauterine growth restriction, maternal thyroid disease, congenital malformations, chromosomal/genetic abnormalities, antepartum hemorrhage.

Hypoxia-ischemia can also affect infants during the intrapartum period. Numerous complications are recognized, such as excessive bleeding from the placenta, very low maternal blood pressure, umbilical cord accidents, prolonged late stages of labor, abnormal fetal position, rupture of

the placenta or the uterus, general anesthesia, emergency cesarean delivery, chorioamnionitis and maternal fever.

Asphyxia can also occur in the immediate postnatal period and premature babies are particularly at risk of suffering brain injury or even death if HIE occurs after delivery. The predominant form of brain damage is white matter leukomalacia, the usual antecedent of cerebral palsy. The most common causes of postpartum HIE are usually secondary to pulmonary, neurological or cardiovascular abnormalities and involve: severe cardiac or pulmonary disease, infections including sepsis and meningitis, severe prematurity, low neonatal blood pressure, brain or skull trauma, congenital brain malformations. In some cases, causes for neonatal asphyxia cannot be identified.

The clinical condition of asphyxia is characterized by the combination of oxygen lack, hypercapnia, acidosis, and ischemia (Volpe, 1995). The word asphyxia means “without pulse” which is the final consequence of severe oxygen lack. It results in neuronal injury of specific regions of the cerebral cortex, hippocampus, thalamus, brain stem and cerebellum (Hill, 1991).

1.1.2. Pathophysiology of hypoxic-ischemic brain injury

The interruption of blood supply to the brain, which leads to insufficient oxygen and glucose delivery, triggers a cascade of biochemical events that cause cell dysfunction and ultimately cell death. Different key metabolic perturbations arising during and after neonatal HI insult have been unraveled using rodent models (combination of hypoxemia and ischemia) which replicate brain damage seen in human infants after asphyxia. The proposed pathogenesis of HIE is demonstrated on Figure 1.

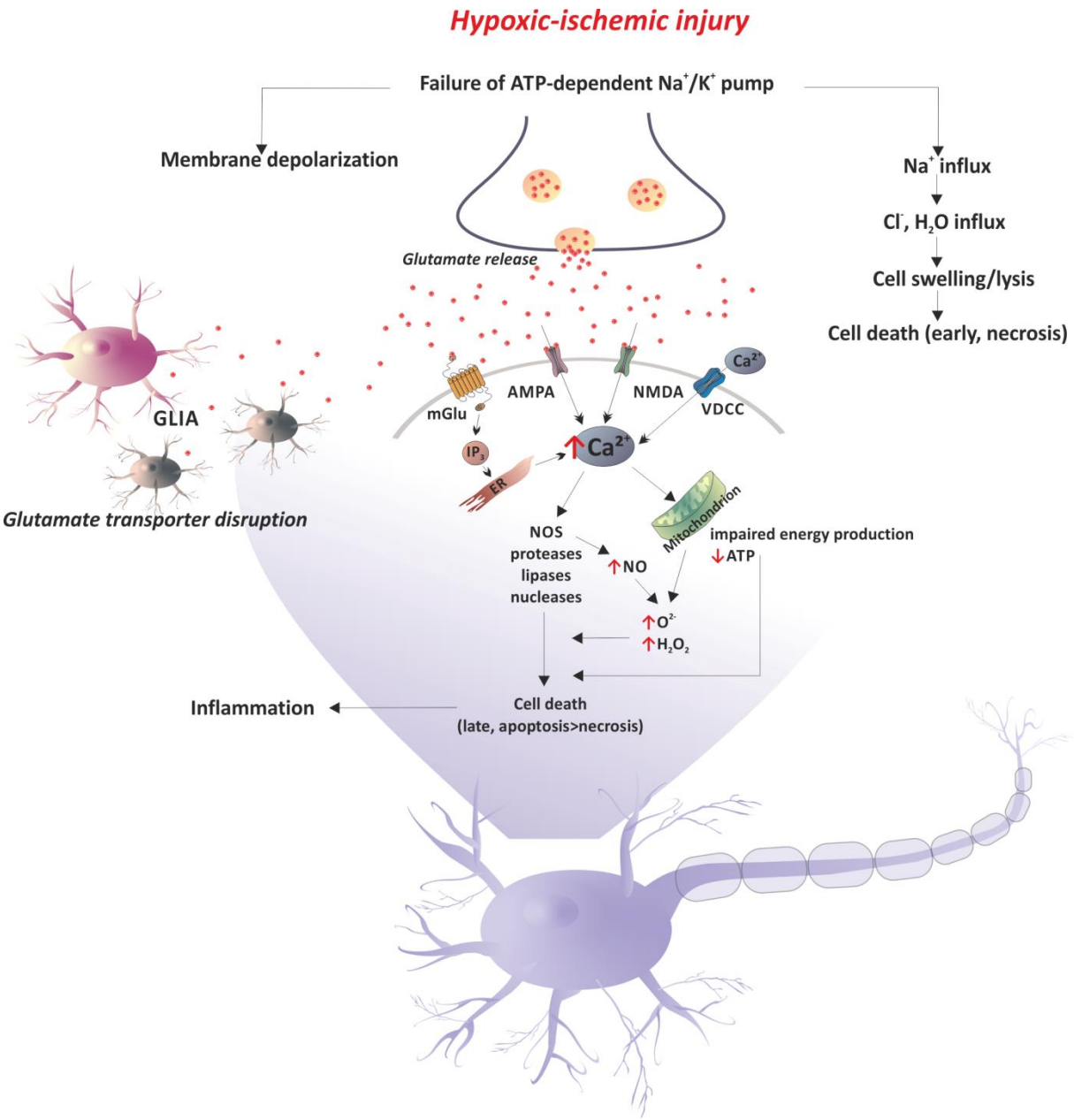


Fig. 1. Mechanisms involved in hypoxic ischemic encephalopathy (Zalewska et al., 2015 -modified).

A depletion of oxygen and glucose precludes oxidative phosphorylation. The decrease in high-energy phosphorylated compounds (ATP and phosphocreatine) below critical threshold initiates a series of additional reactions, beginning with a failure of the ATP-dependent Na^+-K^+ pump. Transcellular ion pump failure in severe insults causes an acute influx of Na^+, Cl^-, and water with consequent cell swelling, cell lysis, and thus early cell death by necrosis in more critical cases. Less severe insults cause membrane depolarization and excessive release of excitatory neurotransmitters, especially glutamate, from axon terminals. Over-stimulation of glutamate receptor/channel complexes triggers calcium influx into the cell, its release from the internal stores and then cascade of Ca^{2+}-mediated intracellular events. Among them are: proteolytic degradation of cellular proteins,

cellular lipids by activation of phospholipases, and DNA by activation of nucleases, as well as an indirect mechanism of destruction mediated by the generation of free radicals and nitric oxide (NO) leading to mitochondrial dysfunction. As a consequence, mitochondrial dysfunction ultimately activates pathways of apoptotic or necrotic cell death. Apoptotic cell death is believed to occur when energy supplies are not completely exhausted, while necrotic cell death takes place when energy supplies are not available (Lai and Yang, 2011).

Both clinical and experimental observations demonstrate that HIE is not a "single" event but is rather evolving process divided into different phases (see rev. Hagberg et al., 2015). The temporal aspects of changes after HI have been identified and include primary and secondary energy failure. Cerebral HI of sufficient severity to deplete tissue energy reserves (primary phase) is often followed by near complete restoration of glucose use, mitochondrial respiration and high energy phosphates after reperfusion and reoxygenation (latent phase) (Gilland et al., 1998). Thereafter, a secondary phase occurs encompassing a depletion of high energy reserves accompanied by cell demise, often referred to as secondary brain injury or secondary energy failure (Azzopardi et al., 1989; Blumberg et al., 1997). The specific mechanisms of secondary damage are only partly understood, but excitotoxicity, mitochondrial impairment, intracellular dysregulation of Ca^{2+} homeostasis, oxidative and nitrosative stress, deficiency of trophic factors and inflammation are all implicated in the process of altered neurogenesis and synaptogenesis (Hagberg et al., 2014, 2015; Thornton et al., 2012). Furthermore, cell death appears critical in the execution phase of cellular demise. Less is known about the mechanism of long-term cell injury and repair (tertiary phase) (Fleiss and Gressens, 2012). According to a report by Bennet et al. (2012) the post-injury time-course can be divided into a latent (O-6 hours), secondary (6-72 hours) and tertiary phase (>72 hours). During the tertiary phase, neurons and glia are lost due to chronic loss of trophic factors, loss of synaptic input from neighboring cells, and loss or failure of new progenitor neural stem cell and glial progenitor cell recruitment (Barrett et al., 2007; Stone et al., 2008).

1.1.3. Neuroinflammatory response

It is generally accepted that one of the leading pathogenic factors of neonatal brain damage is inflammation induced by activation of the central and peripheral immune system.

Immune responses are induced within minutes and can expand for weeks and even months after the insult with specific contribution of different cell types (microglia/macrophages, astrocytes, mast cells). It is clear, however, that the ischemia-induced inflammatory response in the brain is driven primarily by microglial cells acting in concert with other infiltrating immune effectors (Benakis et al., 2015). Under physiological conditions microglia exist in a resting ramified state. The highly motile processes of these cells actively surveil the milieu, constantly sensing changes in

microenvironment, and thus are able to maintain CNS homeostasis. Upon a pathological insult ramified microglia proliferate and become gradually “activated” with shorter processes, migrate to the site of injury and elicit an immune response (Liu and McCullough, 2013). During the progression of HI injury, initially phagocytic, clearing debris and assisting in tissue regeneration microglia (identified as M2 phenotype, called “healthy”) are transformed into M1-type of cells (“sick”), which are known to exacerbate neuroinflammation by the release of pro-inflammatory mediators and recruitment of peripheral immune cells such as monocytes/macrophages. The stimuli-dependent microglial/macrophage immune response is mediated by various signaling pathways through signal transducers and transcription factors (Nayak et al., 2014; Ransohoff and Perry, 2009). These interact with epigenetic moieties to induce chromatin remodeling and transcriptional activation of genes involved in eliciting an immune reaction (Christova et al., 2007).

HI-induced inflammation may result in injury to axons and myelin, loss of oligodendrocyte progenitors, mature oligodendrocytes and neurons (Yong and Marks, 2010). The pathophysiological role of the inflammatory response has been supported by some clinical studies showing an increase in cytokine and chemokine serum levels in newborns with ischemic brain injury. Elevated levels of IL-6 and IL-8 found in the cerebrospinal fluid of term newborns have been correlated with an increased severity of encephalopathy and poor neurodevelopmental outcome (Sävman et al., 1998). Moreover, it is thought that the overexpression of inflammatory molecules might be associated with both cerebral damage entity and prognosis (Aly et al., 2006; Leviton and Dammann, 2004). Both activated intrinsic (microglia) and infiltrating cells (mast cells, monocytes and macrophages) produce several pro-inflammatory mediators – cytokines, chemokines, reactive oxygen and nitrogen species, excitatory amino acid agonists, and death receptor agonists, which could affect brain development with long-lasting consequences causing neurological and/or mental health disorders (Bona et al., 1999; Hagberg et al., 2015; Liu and McCullough, 2013). Consistent with this, there is overwhelming experimental evidence that the reduced microglial activation is associated with diminished brain damage (Arvin et al., 2002; Dommergues et al., 2003).

The reactive astrocytes also appear to be a part of the developing ischemic brain injury (Sen and Levison, 2006; Sullivan et al., 2010). Immediately after the HI insult astrocytes are activated, undergo morphological changes (hypertrophy), proliferate, and form the glial scars in the damaged HI hemispheres (Jaworska et al., 2017; Sullivan et al., 2010). However, the role of reactive astrogliosis in the evolution of ischemic brain lesion, especially in neonates, is at present unclear. In contrast to the potential pro-inflammatory cytokine release, astrocytes are also known to be source of anti-inflammatory mediators. Therefore, it seems reasonable to suppose dual and opposite roles of astrocytes after neonatal HI. The relative contribution of pro-inflammatory and anti-inflammatory mechanisms in astrocytes depends probably on the severity of the insult and the time of recovery.

1.1.4. Current and potential therapeutic strategies for neonatal hypoxic-ischemic encephalopathy

Brain damage following hypoxic-ischemic insults is a complex process which develops over several hours to days and by this it provides an opportunity for therapeutic interventions in the sequence of intracellular events induced by HI. The goal of a neuroprotective therapy is to prevent injury progression, salvage and protect the cells that would otherwise be injured or die, repair the injured cells, and enhance neurogenesis and angiogenesis with the long-term goal of improving neurodevelopmental outcomes. In this context pharmacological intervention aimed at preventing neuronal death represents one of the therapeutic strategies.

1.1.4.1. Pharmacological therapy

To date a number of potential neuroprotective drugs targeting different pathways leading to irreversible brain injury have been tested in animal models of perinatal brain injury. However, despite the beneficial effects of several agents in experimental HI they have not been reproduced in the systemic manner in human infants due to their incapability to repair the damage and/or due to secondary undesirable effects which may influence normal brain function (see rev. Zalewska et al., 2015).

Among the complex chain of events leading to irreversible brain injury free radicals (reactive oxygen and nitrogen intermediates) appear to play a critical role. It is worth underlining that the immature human brain is particularly susceptible to oxidative stress (Ikeda et al., 2002). Therefore, research efforts are focused on agents which will prevent free radical generation and brain damage after perinatal asphyxia. Several antioxidants or free radical scavengers (such as dipyramidol, apotransferrin, vitamin E, allopurinol, melatonin and thiol-containing compounds -glutathione and N-acetyl cysteine) appeared particularly interesting as neuroprotectants in the newborn. Experimental results have shown that they have the capacity to attenuate white matter damage and induce remyelination processes (Farinelli et al., 1998; Guardia Clausi et al., 2012). The cumulative evidence has pointed to a powerful antioxidant and anti-inflammatory agent - melatonin, because of its efficacy and safety profile. However, there is no consensus in the optimal dosing (Robertson et al., 2013) and investigation has been carried out in the last decade to identify optimal treatment doses for full-term babies (e.g. MIND- NCT00649961).

The agents most widely used for treatment of neonates with HIE are antiepileptic drugs. They control directly the seizures associated with HI damage. The anticonvulsant agent Topiramate administered to neonatal rodents immediately after HI increases the seizure threshold, provides protection against white matter injury and is implicated in improving neuromotor deficits (Follett et al., 2004). A phase II trial (NeoNATI-NCT01241019) as well as a phase I/II study of Topiramate

(NCT01765218) have been completed. Although a number of antiepileptic drugs, such as compounds reducing excitatory and/or stimulating inhibitory neurotransmission (e.g. antagonists of NMDA receptor and agonists of GABA A receptor, respectively), as well as known sodium channel blockers (phenytoin, valproate), exert sedative or anticonvulsant effects that are beneficial in neonatal intensive care units, there are also several issues remaining with regard to their possible negative effects.

A great deal of attention has been drawn to neurotrophins, because of their role in transducing survival signals. It has been documented experimentally that Brain-Derived Neurotrophic Factor (BDNF) administered just before the induction of HI protected from 90% tissue loss, whereas applied after the insult protected from 50% of tissue degeneration (Cheng et al., 1997). BDNF pretreatment also prevented spatial memory dysfunction (Almli et al., 2000). However, as this neurotrophin does not cross the blood brain barrier (BBB), it would have to be delivered intracerebroventricularly (ICV), which limits its use as a neuroprotectant due to safety concerns. Other studies emphasized the beneficial role of GDNF, which administered in the form of encapsulated GDNF-secreting cells after experimental neonatal HI protected endangered neural tissues and prevented apoptosis. These preclinical findings present a novel approach toward the treatment of HI encephalopathy in infants.

Cannabinoids and corticosteroids are also considered as promising, clinically applicable therapeutic agents for the treatment of brain damage caused by HI, but it is still ahead of time to perform a clinical trial, despite that corticosteroid therapy has been widely used antenatally to prevent neonatal respiratory distress syndrome.

The only standard care for neonatal hypoxic-ischemic injury in term infants is hypothermia, being embodied in numerous neonatal intensive care units in developed countries. Potential multifold mechanism of neuroprotection is associated with interruption of the destructive pathways that lead to brain damage. Hypothermia reduces cerebral metabolic rate and prevents energy failure. It also inhibits glutamate release, restores pro- and anti-oxidant balance, and inhibits apoptosis (Gluckman et al., 2005; Sameshima and Ikenoue, 2013). Despite the promising outcome of some trials it occurred that hypothermia has been efficient in term-born infants presenting a moderate degree of injury. It is worth underlining that the rate of death or disability in infants with moderate to severe HIE after cooling reaches 46%. Thus, identification of specific diagnostic markers for patients likely to benefit from hypothermia therapy might allow for more targeted selection and improved outcomes.

The potential add-on therapy involving hypothermia combined with inhaled xenon is currently being tested or has been recently completed in clinical trials (NCT02071394 and NCT00934700). Results of these studies are awaited.

Another drug which appeared to be a particularly interesting candidate for clinical application in the near future is erythropoietin (Fan et al., 2010). Clinically, a pilot study of 45 patients showed that treatment of asphyxiated newborns with recombinant human Erythropoietin (rhEPO) seemed safe and feasible (Elmahdy et al., 2010). On the other hand, no beneficial effect of rhEPO treatment on the volume of injury was observed in 21 neonates with diagnosed perinatal arterial ischemic stroke (Benders et al., 2014). Currently, several neuroprotective agents are included in different phases of clinical trials as is shown in Table 1. The results of histone deacetylase inhibitor (HDACi) treatment as a potential therapeutic approach are described in chapter 1.2.3.1. and 1.2.3.2.

Intervention	Included Phases	Number of trials
Hypothermia (head/whole body cooling)	1-3	9
Erythropoietin	1-2	4
Erythropoietin with hypothermia	1-3	5
Autologous umbilical cord blood cells	1	2
Autologous umbilical cord blood cells with hypothermia	1-2	3
Autologous Cord Blood and Human Placental Derived Stem Cells	2	1
Autologous hematopoietic cells (CD34+)	n/a	1
Umbilical Cord-Derived Mesenchymal Stem Cells	1	1
Umbilical Cord Milking	n/a	2
Neural Progenitor Cell and Paracrine Factors of human mesenchymal stem cells	n/a	1
Hyperbaric oxygen	n/a	1
Melatonin	2	1
Cerebrolysin	2	1
Citicoline	3	1
Ascorbic Acid and Ibuprofen	n/a	1
Allopurinol sodium	3	1
Topiramate with hypothermia	1-2	2
Melatonin with hypothermia	Early Phase 1	1
Dexmedetomidine with hypothermia	Early Phase 1	1
Clonidine with hypothermia	1- 2	2

Magnesium Sulphate with hypothermia	3	1
Allopurinol with hypothermia	3	1
Xenon gas with hypothermia	1-2	3
5% carbon-dioxide with hypothermia	1	1
Hydrocortisone with hypothermia	2-3	1
Vitamin E and C	4	1
Behavioral: Infant stimulation	2	1

Table 1. Therapies undergoing clinical trials in human infants with hypoxic-ischemic encephalopathy. Abbreviation: *n/a – not available*. (source: *https://clinicaltrials.gov/)*

1.1.4.2. Cell-based therapy

The lack of effective neuroprotective treatments for newborns suffering from hypoxic-ischemic encephalopathy brings about increased interest in alternative methods, such as regenerative medicine using stem cells, all the more because neonatal HI brain injury induced in rodents stimulates neurogenic response (Kadam et al., 2008). This intriguing discovery led to the speculation, that the central nervous system mounts an intrinsic regenerative potential to repair itself due to persistent production of neural progenitor cells. No wonder that this possibility brings hope for neonates affected by hypoxia-ischemia. It was supposed that the HI-induced enhanced proliferation of neural progenitors would be followed by the migration of the newly generated cells toward the injured brain areas, where they would acquire the desired phenotype enabling the damaged brain area to be reconstructed. However, it occurred that the premature brain is not capable to completely regenerate itself. Due to extensive cellular demise the capacity of endogenous regeneration proved to be rather limited and insufficient for replacing the lost neurons. The majority of proliferating cells died by apoptosis and only 15% of *de novo* produced neurons in the brain cortex have been found 5 weeks after HI (Ong et al., 2005).

Furthermore, it was suggested that transplantation of stem cells may represent a favorable way to stimulate endogenous neurogenesis. A variety of stem cell approaches have been proposed for treatment of HIE. Recently, a safety and feasibility study using autologous human umbilical cord blood cells for neonatal encephalopathy in the first 14 days of life was completed at Duke University USA (NCT00593242). Autologous transplantation of cord blood is promising, as minimal *ex vivo* manipulation is needed, there is no necessary immunosuppression, it is quite easy to obtain and store. Meanwhile, a new therapeutic approach which combines the infusion of umbilical cord blood with erythropoietin has been employed at Sung Kwang Medical Centre (Republic of Korea) for children suffering from cerebral palsy.

In spite of the findings indicating the strong potential of cell-based interventions, much research still needs to be done for developing the best strategy considering transplantation timing, cell dosage, ex vivo modulation, way of administration, and choice of stem cells before translating the results to clinical treatment of neonatal HI.

Hence, alternative approaches for transplantation of exogenous cells are being sought to amplify the endogenous regenerative capacity by introducing molecules that may expand the stem cells pool and maintain their survival. In line with this, mounting evidence indicates that some agents exert stimulation of brain neurogenesis by epigenetic mechanisms, which might function as sensors of rapidly changing environments and valuable modulators of neurogenesis in brain strategic areas (Covic et al., 2010). It was discovered that the epigenetic regulation of gene expression plays an important role in the development and differentiation of the nervous system, and in the maintenance and survival of neurons. In particular the histone acetylation machinery has been found to be an integral part in the crucial aspects of neuroprotection/neurogenesis. Considerable attention has been drawn to histone deacetylase inhibitors. Several groups have reported that treatment with various HDACis, prior or following experimental stroke induction confers generation of new neuronal cells and neuroprotection. The detailed characterization of deacetylase inhibitors is described in the next chapter.

1.2. EPIGENETIC REGULATION OF CELL FUNCTIONING

The term epigenetics ("beyond genetic" or "other than genetic"; the prefix epi – being derived from Greek for "over" or "above") has been introduced by Conrad Waddington in 1942. Epigenetics is most commonly defined as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al., 1996). This definition, however, is not particularly well suited for the nervous system in which there is an overall absence of mitosis. Adrian Bird (2007) proposed a more recent definition of epigenetics as "the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states". In this definition, the three major levels of epigenetic changes are embodied: 1) chemical modifications at the level of nucleotides, which include DNA methylation and RNA interference (RNAi); 2) modifications at the level of histones that encompass posttranslational modification of histone proteins and the incorporation of histone variants; and 3) nucleosome remodeling which refers to ATP-dependent processes that regulate the accessibility of nucleosomal DNA (Fig.2). Epigenetic regulation represents a fundamental mechanism to maintain cell-specific gene expression ranging from modulation of normal embryonic development to plasticity in the adult nervous system, as well as the maintenance of neurons. Among the above mentioned epigenetic changes, modifications of histone proteins are emerging as essential mechanisms regulating many important cellular processes including the expression of genes required for neuronal development.

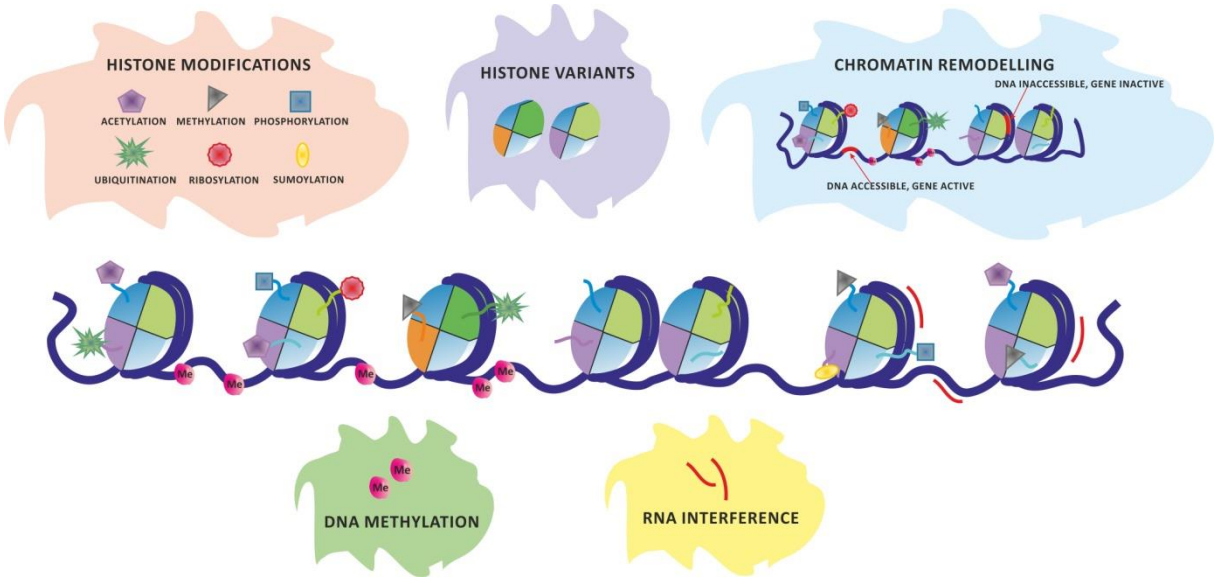


Fig. 2. Overview of possible epigenetic modifications. All types of epigenetic changes (histone modifications and variants, chromatin remodelling, DNA methylation and RNA interference) regulate the accessibility of the chromatin structure to the transcription machinery (Gräff et al., 2011 - modified).

1.2.1. Epigenetic modifications of histones

The N-terminal tails of histones are subject to at least six distinct post-translational modifications, including acetylation, methylation, ubiquitination, phosphorylation, ribosylation, and SUMOylation. These histone tail amino-acid specific modifications, named “histone codes” regulate the genomic accessibility and provide a platform for binding of other factors to control the activation or repression of associated genes. The most intensively studied type of histone modification is a coordinated process carried out by two classes of enzymes – histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes are major players in epigenetic mechanisms regulating transcription and other functions in various cells including neurons, microglia, and astrocytes. HATs catalyze the transfer of an acetyl group from acetyl-coA to the ε-amino site of lysine, neutralizing the positive charge on histones. Histone acetylation provides a more open chromatin structure, correlating with gene transcriptional activity. Moreover, this unfolding of chromosomal domains also enhances the process of transcriptional elongation. Conversely, a decrease in acetylation (hypoacetylation) may cause compressing of the DNA/histone complex, restricting access of transcription factors to the DNA and repressing gene expression. Thus, HATs and HDACs provide the enzymatic basis for transcriptional activation and repression, respectively, through alteration of the chromatin structure. Direct acetylation and deacetylation of transcription factors has also been shown to have positive and negative consequences on gene transcription. Therefore, the balance of these two groups of enzymes is a key element in the regulation of specific sets of genes and is crucial for cell proliferation, differentiation and homeostasis. Under normal conditions, protein concentration and enzymatic activity of HATs and HDACs remains in a highly harmonized state of balance where adequate active molecules from either group are present to effectively regulate chromatin and transcription factor acetylation in a controlled manner. Such equilibrium manifests neuronal homeostasis and is responsible for regulated gene expression providing normal neurophysiological functions (Hull et al., 2016; Shein and Shohami, 2011).

1.2.2. Overview of the histone deacetylase (HDAC) family

Histone deacetylases (HDACs) are evolutionary conservative enzymes that are found in all eukaryotic cells. Two categories of HDACs have been identified thus far: the “zinc-dependent” ones and the “nicotinamide-adenine-dinucleotide (NAD)-dependent” sirtuins. HDACs exist in multiprotein complexes with transcription factors, DNA-binding proteins and other chromatin modifying enzymes. Their assembly into complexes is required for full deacetylase activity.

In mammals, up to date 18 deacetylases have been identified (Table 2). Depending on sequence similarity, the zinc-dependent HDAC family members are categorized into 4 different classes (Class I, IIa, IIb and IV), which differ in structure, enzymatic function, subcellular localization

and expression patterns. In addition to these classical HDACs, mammalian genomes encode another group of deacetylases, the sirtuins (SIRTs), which are sometimes referred to as class III HDACs.

Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are expressed ubiquitously, localized predominantly to the nucleus and display high enzymatic activity towards histone substrates. It is worth to point out that HDAC1 and HDAC2 are the best characterized deacetylases among all HDACs. Class II HDACs further subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) can shuttle from the nucleus to the cytoplasm. Of note, only HDAC6 is predominantly cytoplasmic. In some cases class IIa HDACs can also act as transcriptional activators, but in either situation these enzymes primarily control gene expression by recruiting other proteins (corepressors or coactivators). Class IV currently consists of one member, HDAC11, with little knowledge of its function. Class IV has a unique catalytic domain and is overall structurally distinct from class I and II HDACs. The sirtuins, which have an uncommon property in that they require NAD^+ as a cofactor for their enzymatic activity, are composed of 7 members (SIRT1 to SIRT7) with distinct cellular localization. All the sirtuins present higher expression in fetal brain and may play crucial roles in early brain development (Hull et al., 2016).

	Co-factor	Subcellular localization
Class I		
HDAC 1	**Zn^{2+}**	***Nucleus***
HDAC2		***Nucleus***
HDAC3		***Nucleus/Cytoplasm***
HDAC8		***Nucleus***
Class IIa		
HDAC4	**Zn^{2+}**	***Nucleus/Cytoplasm***
HDAC5		***Nucleus/Cytoplasm***
HDAC7		***Nucleus/Cytoplasm***
HDAC9		***Nucleus/Cytoplasm***
Class IIb	**Zn^{2+}**	
HDAC6		***Cytoplasm***
HDAC10		***Cytoplasm***
Class III sirtuins		
Sir 1		
Sir 2		***Nucleus***
Sir 3	**NAD^{+}**	***Nucleus/Cytoplasm***
Sir 4		***Mitochondria***
Sir 5		***Mitochondria***
Sir 6		***Mitochondria***
Sir 7		***Nucleus***
Sir 8		***Nucleolus***
Class IV	**Zn^{2+}**	
HDAC 11		***Nucleus***

Table 2. Categorization of histone deacetylases (Jaworska et al., 2015 - modified).

1.2.2.1. Biological functions of HDACs

HDACs regulate activity of their substrates by removing acetyl groups from lysine residues. Of note, recent phylogenetic study of bacterial HDACs divulged that all four classes of deacetylases preceded the evolution of histone proteins, suggesting that the primary action of these enzymes might have been towards non-histone substrates. These include wide variety of intracellular targets, e.g. transcription factors, transcription regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators.

Biological functions of individual HDACs have been difficult to define due to the lack of isoform-specific inhibitors. This obstacle is related to the high sequence homology within the catalytically active sites of HDACs. Moreover, constitutive knock-out (KO) of many of the individual HDACs are lethal, underscoring the vital role of these enzymes in normal development, but rendering the use of constitutive KO models unsuitable for study in adults. Conditional KO and small interfering RNA (siRNA) strategies are now being employed to study the unique functional profiles of individual HDAC isoforms (Jaworska et al., 2015). Primarily, through the use of non-specific "pan" – HDAC inhibitors which inhibit many or all deacetylases, it appeared that these enzymes are implicated in diverse biological processes, including, but not limited to, tissue specific developmental programming, apoptosis, synaptogenesis, cognition, cancer, ischemia, and neurodegenerative diseases (Haberland et al., 2009; Minucci and Pelicci, 2006).

A substantial body of evidence has documented the necessity of HDACs function for normal developmental processes and tissue patterning by silencing the expression of specific growth-inhibitory genes (Brunmeir et al., 2009; Lagger et al., 2002). To support this, it has been indicated that constitutive deletion of certain isoforms of class I HDACs, e.g. HDAC1 or HDAC3 causes several developmental defects and/or lethality (Haberland et al., 2009; Lagger et al., 2002).

1.2.2.2. Histone deacetylases in the nervous system

All isoforms of HDACs class I, II and IV are expressed in the brain (Broide et al., 2007). Studies performed in zebrafish and, more importantly, in small mammals revealed particularly important functions of two deacetylases, HDAC1 and HDAC2, in the central nervous system development (Jaworska et al., 2015). Expression of both isoforms in neural progenitors and stem cells embryonically and postnatally, therefore in multiple levels of maturation and tissue patterning, supports their involvement in the regulation of neurogenesis (Humphrey et al., 2008; MacDonald and Roskams, 2008; Montgomery et al., 2009). The inhibition of normal HDAC function, at certain time periods during embryonic evolvement, shifts the fate of neural progenitors from glial to neuronal and strongly implicates a role for deacetylase enzymes in cell-fate decisions in the brain. The highly homologous HDAC1 and HDAC2 appear to be segregated to distinct stages of neuronal and glial lineages during CNS development and their expression pattern suggests that they may modulate distinct gene expression events at different developmental stages (MacDonald and Roskams, 2008). Neural progenitors that maintain the expression of HDAC1 largely differentiate into glial cells, while those that lose HDAC1 and upregulate HDAC2 differentiate into neural progenitors and neurons, as illustrated on Fig. 3.

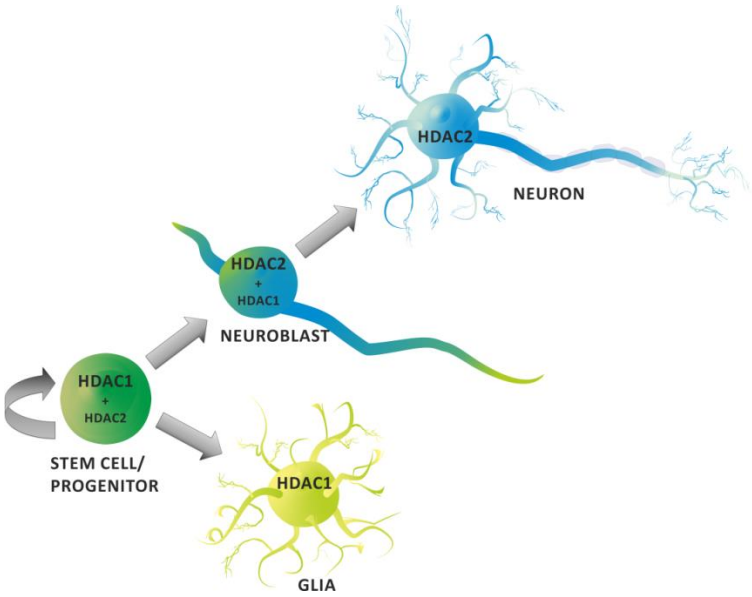


Fig.3. Involvement of HDAC1 and HDAC2 in neural development (Jaworska et al., 2015 - modified).

It was suggested that HDAC1 is a critical regulator of the production and differentiation of oligodendrocyte precursor cells (Marin-Husstege et al., 2002; Shen and Casaccia-Bonnefil, 2008). HDAC1 can also directly regulate stem cell proliferation, and animals devoid of this isoform display a significant reduction in cell division (Lagger et al., 2002). HDAC2, on the other hand, appears necessary to inhibit astrocyte differentiation (Humphrey et al., 2008), suggesting that this enzyme may be involved in silencing glial gene expression. On the contrary, HDAC1 likely silences neuronal genes. It is becoming clear that an extensive number of developmental decisions and differentiation programs depend on HDAC1 and HDAC2 as co-factors. In accordance with this HDAC1/2 DKO severely disrupts cortical, hippocampal and cerebellar organizations and the mice do not survive beyond postnatal day 7 (Montgomery et al., 2009). This implicates their contribution to the regulation of the developmentally specific gene expression and to maintenance of the CNS.

Considering the potential role of HDAC in these processes it should be remembered that histones are not the sole target or substrate of HDAC1 and 2. Several HDAC isoforms have been shown to regulate acetylation of a plethora of proteins, which also participate in neurodevelopment. Thus, specific modification of a great number of HDAC substrates indicates the involvement of deacetylation in a whole array of biological processes, including among others regulation of neurogenesis, apoptosis, synaptogenesis, and neurite outgrowth. Once the balance between acetylation/deacetylation is disturbed by reduction or loss of histone acetyltransferases, the HAT:HDAC ratio tilts in favor of HDAC in terms of enzymatic functionality leading to a loss of acetylation homeostasis, as is observed during several neurological disorders (Dinarello et al., 2011), including cerebral ischemia induced in adult (Kassis et al., 2016; Kim et al., 2009; Ren et al., 2004) as

well as in neonatal rats (Koyuncuoglu et al., 2015). Current data suggest that histone deacetylase inhibitors could possibly correct aberrant acetylation patterns associated with neurological impairments and ameliorate the disease state. In line with this, inhibition of HDACs is introduced as a therapeutic strategy in several models of neurodegeneration (Haberland et al., 2009; Langley et al., 2009; Selvi et al., 2010).

1.2.3. Histone deacetylase inhibitors (HDACis)

Histone deacetylase inhibitors (HDACis) are a heterogeneous group of agents that inhibit histone deacetylases and promote posttranslational acetylation of lysine residues within nuclear and cytoplasmic proteins, which may alter their activity and function. In particular, HDAC inhibition can have a profound effect on the acetylation status of histone proteins within the chromatin, resulting in augmented expression of genes relevant to protection from an ischemic insult. In addition, inhibition of deacetylation equally promotes the acetylation of non-histone proteins, such as transcription factors, signal transduction mediators, determining their interaction, localization and stability (Glozak et al., 2005).

To date, numerous HDACis have been synthesized or isolated as natural products which have various target specificity, pharmacokinetic properties and activities in laboratory and clinical settings. The chemical classes of HDACis include inhibitors of both zinc-dependent (classical HDACs) and NAD^+ -dependent HDACs (SIRT inhibitors). This chapter will be focused on inhibitors of the classical HDAC family, which function by chelating the zinc ion at the deacetylase active site.

HDAC inhibitors of the classical deacetylase family can be grouped into diverse classes based on their chemical structures: aliphatic acids, hydroxamic acid dervatives, benzamides, cyclic peptides and a few substances not assignable to these groups. The most commonly used HDACis are presented in Table 3. Most HDACis are broad-spectrum and affect multiple HDACs and thus are known as “pan-HDAC inhibitors”. Unfortunately, isoform-specific inhibition of HDACs still remains a challenging task. In many cases these compounds exhibit a high degree of lipophilicity and are difficult to design due to the high sequence homology within the catalytically active sites of HDACs, rendering them more useful as tools for research (Dallavalle et al., 2012). Only a few newly characterized HDAC inhibitors are now available that preferentially inhibit specific HDAC classes, such as CHR-3996 (inhibits class I HDACs). In addition, selective inhibitors have been developed for HDAC6.

Classification	Examples	Specificity to HDAC
Aliphatic acids	Butyrate/ Sodium Butyrate (SB, NaB)	***Class I/IIa***
	Phenyl Butyrate (PB)	***Class I/II***
	AN-9 (Pivaloyloxymethyl Butyrate)	***Class I***
	Valproic acid (VPA)	***Class I/IIa***
Hydroxamates	Suberoylanilide hydroxamic acid (SAHA; vorinostat)	***Class I/II/IV***
	Trichostatin A (TSA)	***Class I/II***
	PDX-101 (belinostat)	***Class I/II/IV***
	LBH-589 (panobinostat)	***Class I/II/IV***
	ITF2357 (givinostat)	***Class I/II***
	4SC-201 (resminostat)	***Class I/II***
	PCI-24781 (abexinostat)	***Class I/II***
	Tubacin	***HDAC6***
	NVPLAQ-824 (dacinostat)	***Class I/II***
Benzamides	MS-275, SNX-275 (entinostat)	***HDAC1/9/11***
	MGCD0103 (mocetinostat)	***HDAC1/2/11***
	CI-994 (tacedinaline; N-acetyl dinaline)	***HDAC1/2***
	Rocilinostat (ACY-1215)	***HDAC6***
	Chidamide (Epidaza)	***HDAC1/2/3/10***
Cyclic peptides	Depsipeptide (FK228; romidepsin; FR901228; isostax)	***HDAC1/2***
	Apicidin	***HDAC2/3***
	Depudecin	***Class I/IIa***
	Trapoxin B	***HDAC1/4/11***

Table 3. The most common histone deacetylase inhibitors (Tandon et al., 2016 - modified).

It is very likely that the non-specificity of deacetylase inhibitors is responsible for the opposing effects noted in distinct type of cells. As it is becoming apparent, HDAC inhibition promotes the demise of tumor cells. The same drugs display strong protective properties for neurons in *in vitro* and *in vivo* models of neurotoxicity and neurodegeneration. Similarly, the different effects of HDACis are also observed in cells that contribute to inflammatory pathways, where treatment results in pro- or anti-inflammatory stimulation (Halili et al., 2009). One of the explanations of such discrepancy involves the particular function of individual HDAC isoforms in activation of different transcription factors and then expression of different sets of genes.

1.2.3.1. Histone deacetylase inhibitors as potential neuroprotective agents

Treatment with various HDAC inhibitors has emerged as an attractive therapeutic approach for acute injury and neurodegeneration in the last decade (Haberland et al., 2009; Langley et al., 2009; Selvi et al., 2010). However, one of HDACis – VPA was approved as early as 1978 and is still used as an anticonvulsant and mood stabilizer in the treatment of various neurological disorders.

The attractiveness of HDACis has been increased by the finding that HDAC inhibitors can also enhance neuronal plasticity and memory and thereby may contribute to improved functional and cognitive recovery. Deacetylase inhibitors enhance long-term potentiation (LTP) at hippocampal synapses and also in the amygdala, two brain regions that are essential for associative learning, leading to sprouting of dendrites, an increase in the number of synapses and reinstating long term memories.

A number of studies have demonstrated HDAC inhibitors to be protective in animal models of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, and Experimental Immune Encephalomyelitis. Furthermore, currently several HDACis are included in various clinical and preclinical trials for the treatment of neurological diseases. More recently, inhibitors of histone deacetylases have received considerable attention as emerging tools for therapeutic interventions in the context of post-acute stroke. Substantial research has been focused on potential roles for a number of zinc-dependent HDAC inhibitors (such as TSA, VPA, SB) used in different models of adult brain ischemia. It occurred that treatment of adult animals subjected to brain ischemia with histone deacetylase inhibitors resulted in a significant reduction of infarct volume, suppression of neuroinflammation in the ischemic region, neurobehavioral improvement, and, importantly, stimulation of neurogenesis in association with increased histone acetylation.

1.2.3.2. Potential mechanism of protection in brain ischemia

The potential explanations for the beneficial effects of HDACs inhibition following adult brain ischemia are multifold and according to the published results include influencing a diverse array of targets to maintain neuronal function and to preserve white matter in response to injury. The action of deacetylase inhibition involves, among others, influence on oxidative pathways and/or downstream components of excitotoxicity, and reduced expression of various factors engaged in apoptosis. One of the best described modes of protective action is associated with the reduction of neuroinflammation, as was noted above, by suppressing astrocytic and microglial activation and down-regulating pro-inflammatory factors. Moreover, it was reported that HDACi treatment enhanced post-ischemic neurogenesis and angiogenesis by increasing microvessel density, facilitating endothelial cell proliferation, and increasing the rate of blood flow to the ischemia-affected cerebral

cortex. The newly-generated vessels provide additional neurotrophic support to concurrent neurogenesis and synaptogenesis, and this ultimately may lead to functional recovery. These events, together with the long-lasting neurological improvement, suggest that HDAC inhibition might have utility in treating acute stroke. On the molecular level, HDACi action results in enhanced acetylation of histone proteins with gene promoters and regulatory regions, as well as transcription factors, and by this can increase the expression of multiple genes which protein products contribute to neuroprotection, plasticity and memory. The expression of cytoskeletal proteins has also been implicated in neuroprotection by HDAC inhibition under ischemic conditions. For instance, HDAC inhibition upregulated gelsolin, a protein involved in actin filament organization, and by this route contributed to neuroprotection after ischemic brain injury (Yildirim et al., 2008).

A significant amount of data show that the administration of HDACis after stroke correlated with upregulation of HSP70, a probable viable target for neuroprotection, in addition to being a molecular chaperone assisting in proper protein folding. Anti-apoptotic effects of HSP70 may involve multiple mechanisms, such as inhibition of cytochrom c-dependent activation of death promoting caspase-3 and its downstream effectors and enhanced expression of anti-apoptotic Bcl2 proteins after experimental stroke. It might also act by blocking the action of transcription factors that regulate apoptosis, such as the tumor suppressor p53. Furthermore, inhibitors of HDACs may compensate MCAO induced deficiency of pro-survival phospho-Akt and phospho-ERK in the ischemic hemisphere and significantly raise the level of BDNF and GDNF expression. The increased expression of neurotrophins by HDACi treatment was found to be involved in the post-ischemic neuroprotection and neuronal restorative effect by the recruitment of endogenous progenitors to the site of damage.

Despite mounting evidence concerning the role of HDACis in neuroprotective/neurogenic processes in experimental ischemic models in adults, only a few studies addressed the impact of HDAC inhibitors upon post-HI neurogenesis in the immature brain. These experiments were performed with the use of different experimental models, like ventral hippocampus excitotoxic lesion, lipopolysaccharide–sensitized hypoxic-ischemic brain injury (LPS/HI) in mice, or permanent ligation of the carotid artery, thus it is not possible to make an explicit conclusion regarding the effect of HDACi on the generation of new cells (Fleiss et al., 2012; George et al., 2013; Kabakus et al., 2005).

1.3. NEUROGENESIS

1.3.1. Brief history

Neurogenesis refers to the birth of neurons and is a complex process that begins with the division of a precursor cell and ends with the formation of a fully differentiated, functioning neuron. Until 1998, the limited capacity of the brain to regenerate led to the long-held belief that all neurons were developed before birth in the human brain and their generation was impossible in postnatal life. For many years, there was little or no progress in the field. The first hint for occurrence of adult neurogenesis came in 1912 by Ezra Allen (Allen, 1912). Allen showed mitotic figures in the lateral ventricles of albino rats of up to 120 days of age. However, despite this finding, the famous neurobiologist, Santiago Ramon y Cajal in stated 1913 "In the adult centers, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated," (Ramón y Cajal, 1913). And this was in part a reason for slow progress for decades in the field. The complexity of the neural networks in the adult brain affirmed this view. It was assumed that if new neurons were added they would destabilize the neuronal network and as such, it was impossible to integrate the new cells.

The development of advanced techniques for studying dividing cells rekindled interest for the field and helped to yield a big leap in its progress. Altman and Das (1965) injected adult rats with tritiated thymidine, a nucleoside that is taken up in cells that are synthesizing DNA just before the onset of cell proliferation. They observed newly generated cells postnatally in the dentate gyrus (DG) and subventricular zone (SVZ) and described the migratory path to the olfactory bulb. However, their study was not immediately accepted by the neuroscience community for two reasons. At that time, there was insufficient evidence that the labelled cells were neurons. Secondly, the labelled cells could have been undergoing DNA repair, hence leading to incorrect interpretation of the results. Nearly 20 years later these studies were replicated by Kaplan and Hinds, who used a more advanced imaging technique, electron microscopy. This method allowed them to confirm the presence of proliferating cells in the brain of a 3-month old rat (Kaplan and Hinds, 1977). The morphology of these cells pointed towards a neuronal phenotype. However, these findings were not completely accepted due to solely morphological criteria. The interest for the field was restored in the 1980s. Fernando Nottebohn in 1983 demonstrated that a substantial number of neurons are generated in the song system of adult birds (Goldman and Nottebohm, 1983). This gave evidence for synaptic integration of new neurons in the song system of adult male song birds.

However, despite a growing number of studies, research regarding neurogenesis did not move forward until the development of more sophisticated methodological tools in the 1990s. During the following decade, the identification of stem cells in the adult mouse brain proved that precursor cells divided to produce new neurons and glial cells. In 1994 Heather Cameron and

Elizabeth Gould (Cameron and Gould, 1994) made a third re-discovery of adult hippocampal neurogenesis in rats and later, in 1997, Michael Kaplan extended Altman and Das findings by describing cellular phenotype of neurons in mice. In the meantime, in 1996, researchers found that while the rate of neurogenesis in the brain declined as rats aged, it was never abolished completely (Kuhn et al., 1996). Concurrently, this was the same period bromodeoxyribouridine (BrdU), a nucleoside analog, which labels mitotically dividing cells was developed (Kuhn et al., 1996). The phenomenon of postnatal neurogenesis was then reported in humans by Eriksson et al. (1998). The study used BrdU to label neuronal progenitor cells, and found that majority of cells in the subgranular and granular zones of the DG incorporated BrdU and about 22% of these cells co-expressed a neural antigen. This was a major advancement and a breakthrough in the study of postnatal neurogenesis.

In 2002 researchers at The Salk Institute in California visualized and recorded newborn neurons in living mice, something which hadn't been possible before. The researchers watched as over a period of several months, the new cells grew and began to look and behave like mature neurons, forming connections (synapses) and firing electrical impulses (van Praag et al., 2002).

It was discovered that neurogenesis was tightly regulated by the local environment or the "neurogenic niche" (Alvarez-Buylla and Lim, 2004; Doetsch, 2003). The niche is composed of the extracellular matrix and various cell types, including astroglia, endothelial cells, ependymal cells (in SVZ exclusively), immature progeny of the adult neural stem cells and mature neurons within the local circuitry (Jordan et al., 2007; Ma et al., 2005).

Postnatal neurogenesis occurs in two discrete regions in the mammalian brain – the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles. Whether neurogenesis occurs in brain areas other than SGZ and SVZ still remains controversial. However, many subsequent reports confirmed the existence of neurogenic events in other CNS regions (Bonfanti and Ponti, 2008; Gould, 2007; Migaud et al., 2010).

Adult neurogenesis appears to recapitulate the complete process of neuronal development, ranging from neural progenitor activation and fate determination, to differentiation, migration, and axonal and dendritic development of newborn neurons, to synapse formation and functional integration into the existing circuitry (Duan et al., 2008).

1.3.2. Neurogenesis in the hippocampus

The hippocampus is part of the limbic system, which is involved in a variety of higher order functions including emotion, behavior, and memory. It is well established that the dentate gyrus (DG) in the hippocampus is one of two well-accepted regions with continuous addition of new neurons throughout life (Kempermann et al., 2015). The hippocampal neurogenesis is a complex process that

originates from proliferation of neural progenitor cells (NPCs) located in the subgranular zone (SGZ), a germinal layer between the granular layer and hilus (Fig.4). The progenies are born, migrate a short distance to differentiate into granule cells that project their dendrites into the molecular layer (ML) and axons to the CA3 pyramidal cell layer via the mossy fiber pathway and establish synaptic connection with local neurons (Kempermann et al., 2015; Toni et al., 2008).

There are four types of progenitor cells in the dentate gyrus: radial astrocytes (Type B cells or type I cells), immature dividing cells (type D cells or type II cells), newly generated granule neurons (type G cells or type III cells) and endothelial cells. However, the SGZ niche also hosts many other cells. These other niche elements include excitatory hilar mossy cells, glial cells, and many classes of interneurons (mostly inhibitory) (Freund and Buzsáki, 1996; Masiulis et al., 2011), which are highly integrated into the hippocampal circuitry and influence the proliferation, survival, and maturation of adult-generated cells in the dentate gyrus.

SGZ astrocytes are in close proximity to blood vessels and extend basal processes under the blades of the dentate gyrus and an apical process into the granule cell layer. They are the primary precursors of neurons and express such markers as Glial Fibrillary Acidic Protein (GFAP), Nestin, Sox2, and brain lipidbinding protein (BLBP) but not doublecortin (DCX) and polysialic acid-neural cell adhesion molecule (PSA-NCAM) (Seri et al., 2004; Steiner et al., 2006; Suh et al., 2007). The SGZ astrocytes divide to give rise to immature dividing type-II cells. This stage marks the transition between cells with astrocytic phenotype (type-IIa cells, the early stage of type-II cells) and cells with early features of the neuronal lineage (type-IIb cells, the later stage of type-II cells). A panel of different markers (Sox2, BLBP, DCX, and NeuroD) discriminates between the type IIa and type IIb cells. Type-IIa cells feature, to some degree, properties of radial glia-like cells marked with BLBP and Sox2. NeuroD and DCX, the markers of immature neurons, appear in type-IIb cells and persist into post-mitotic but immature granule cell precursors with transient Calretinin-expression (type-III cells). Therefore, type-IIb cells are committed to the neuronal lineage. The type-III cells exit from the cell cycle and begin the terminal post-mitotic differentiation of granule cells (Kempermann et al., 2004; Steiner et al., 2006). As mentioned earlier, this stage is characterized by the transient expression of calretinin, which is later exchanged for calbindin, present in the mature granule cells, that also specifically express NeuN and Prox1 (Brandt et al., 2003; Kempermann et al., 2004). These newborn granule cells elongate their dendrites and axons integrating into the DG circuitry (van Praag et al., 2002; Song et al., 2005). In an addition to the putative markers described above, other genes are expressed in different stages of hippocampal neurogenesis (Hu, Pax6, TUC-4, Msi1, NeuroD) (Englund et al., 2005; Kaneko et al., 2000; Kawai et al., 2004). SGZ neurogenesis occurs in parallel to angiogenesis and endothelial cells act as scaffolding cells for NPCs. Therefore, endothelial cells provide signals and soluble factors that favor angiogenesis but also neurogenesis.

In the human brain, approximately 700 new neurons are generated in the hippocampus per day (Spalding et al., 2013). To date, hippocampal neurogenesis and cell proliferation have been positively associated with environmental enrichment and exercise, which lead to enhanced synaptic plasticity, learning and memory (Bruel-Jungerman et al., 2005; Deng et al., 2009), while negatively associated with stress and certain brain diseases such as depression and HD (Gil et al., 2005; Lemaire et al., 2000; Pittenger and Duman, 2007).

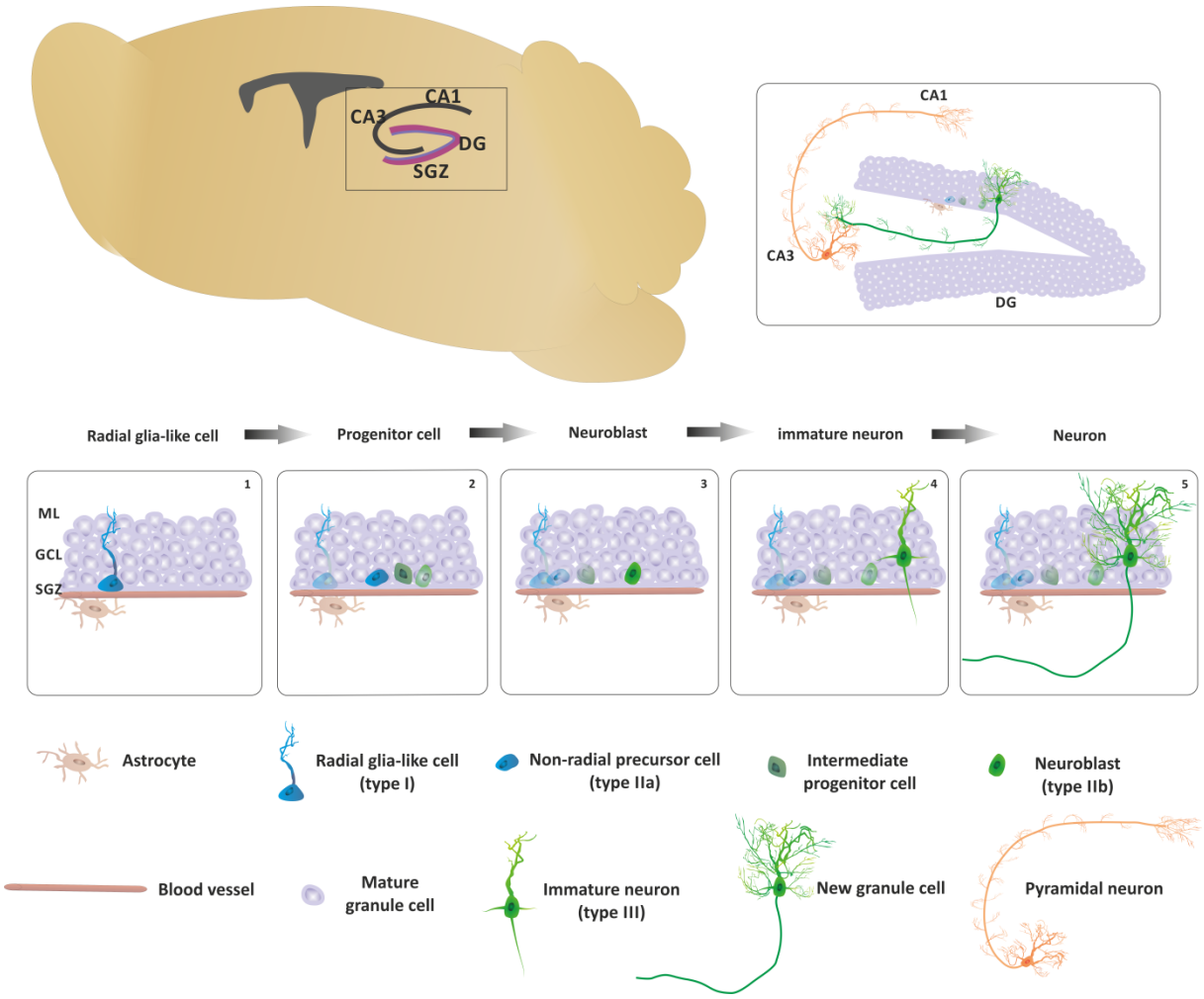


Fig.4. Neurogenesis in the hippocampus (Ming and Song, 2011 - modified).

1.3.3. Neurogenesis in the subventricular zone

The SVZ represents the largest neurogenic stem cell region within the postnatal brain. It resides within a narrow region of the lateral ventricular (LV) wall, roughly four to five cells in diameter. Neural progenitors generated from this region proliferate and give rise to neuroblasts, which then migrate through the rostral migratory stream (RMS) towards the olfactory bulb, and differentiate into granule neurons and periglomerular neurons (Bovetti et al., 2007; Lledo et al., 2006; Lois et al., 1996)(Fig. 5). In fact, it is estimated that 10,000 to 30,000 to 80,000 (Pignatelli and

Belluzzi, 2010) new neurons are generated in the rodent olfactory bulb per day. Differentiation into oligodendrocytes of the corpus callosum white matter also occurs, however to a lesser extent.

There are at least five main different cell types integrating the SVZ: Astrocytes, also called B cells (expressing markers such as GFAP), divided in B1 (apical/quiescent B) and B2 (tangential/active B) based on their expression of markers BLBP (Fabp7), nestin and EGF receptor, which are absent during the quiescent phase and expressed following activation; transit amplifying cells (C cells, the putative precursor); neuroblasts (A cells) and ependymal cells (E cells) (Mirzadeh et al., 2008; Shen et al., 2008). The first layer of cells contacting the ventricular surface is mostly composed of ependymal cells (type E cells) with some intercalated type B1 cells. The primary progenitors of new neurons, astrocytic stem cells (B cells), generate rapidly dividing transit amplifying progenitors, which give rise to neuroblasts that migrate by moving tangentially through the rostral migratory stream (RMS) and into the olfactory bulb (OB) (Lois et al., 1996). This cellular movement within the RMS is called “chain migration”, a term established by Lois and coworkers in 1996. They showed that neuroblasts migrated in clusters without axonal guidance or radial glia regulation using a network of astrocytes that form “glial tubes” (Lois et al., 1996). This unique type of migration is thought to enable cells to draw on neighboring cells as their scaffold for migration (Murase and Horwitz, 2002). The migration is under the control of various signaling molecules, such as polysialated neural cell adhesion molecule (PSA-NCAM), slits, ephrins, integrins, ErbB4, GABA, and different growth factors such as Glial cell line-Derived Neurotrophic Factor (GDNF), Brain-Derived Neurotrophic Factor (BDNF), and Vascular Endothelial Growth Factor (VEGF) (Belvindrah et al., 2007; Chiaramello et al., 2007; Ghashghaei et al., 2006; Hu, 2000; Paratcha et al., 2006). In addition to these molecular cues, cellular interactions between neuroblasts, astrocytes, and blood vessels (BVs) are required for faithful migration toward the OB (Bovetti et al., 2007). However, the astrocyte tubes only appear 2 to 3 weeks after birth. Therefore, migration to other brain regions, such as the striatum, may be facilitated in the first 3 postnatal weeks, as glial tubes may restrict the migration of progenitors within the RMS (Peretto et al., 2005). In the OB the neuroblasts exit the RMS, change direction and migrate radially into the granule cell layer and glomerular layer of the olfactory bulb. The majority of neuroblasts that reach the OB die, with about 40% of newly born cells surviving throughout the life of the animal. Nearly all of the newly born cells differentiate into a neuronal phenotype and functionally integrate into the existing circuitry (Whitman and Greer, 2007b, 2007a). Most of these cells become GABA or Calretinin positive inhibitory interneurons (neurons regulating the firing rate of target neurons), which play very important functions in olfactory processing and transforming odor information. However, a small percentage of these cells become tyrosin-hydroxylase (TH) positive dopaminergic neurons. This “turnover” of cells, represents a ~6–10% replacement of the existing

cellular population within the OB granule cell layer. Granule cells play a role in olfactory discrimination and learning (Gheusi et al., 2000; Rochefort et al., 2002; Schellinck et al., 2004).

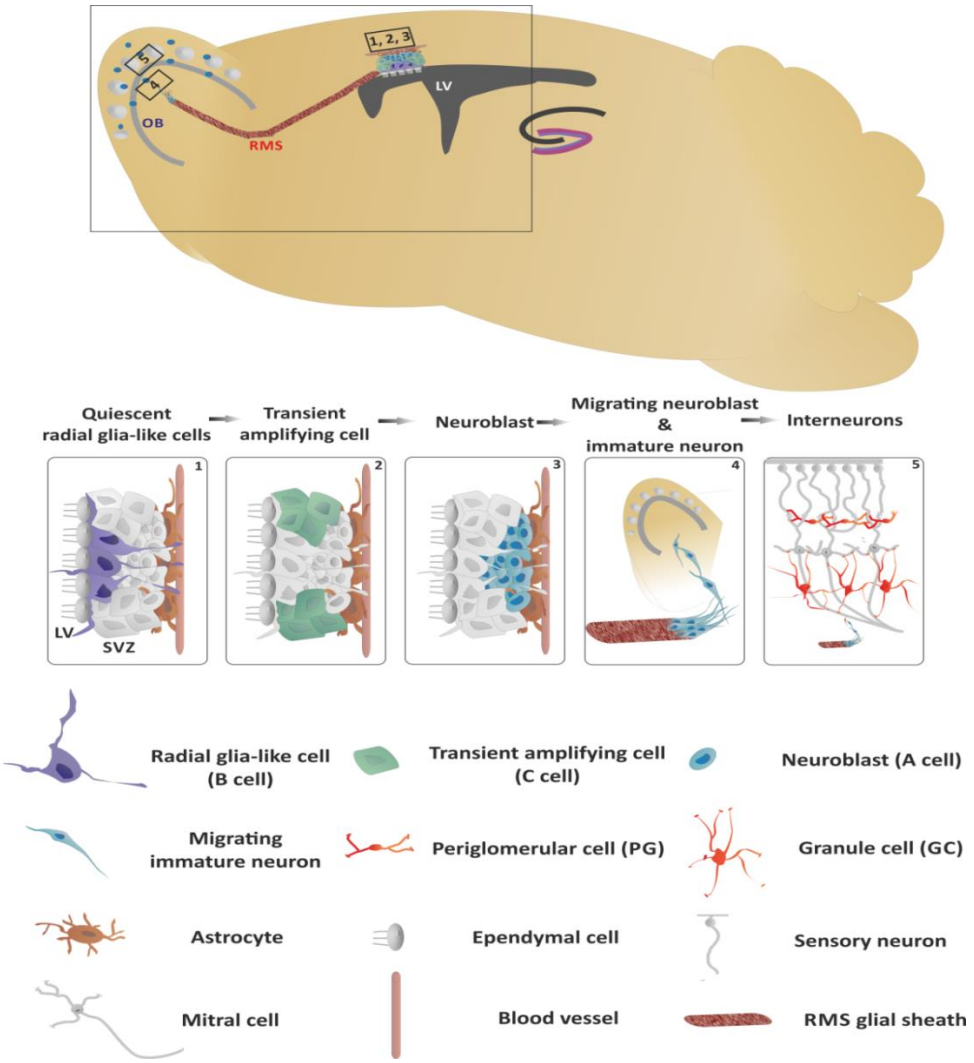


Fig.5. Neurogenesis in the subventricular zone (Ming and Song, 2011 - modified).

1.3.4. Neurogenesis after a hypoxic-ischemic insult

Many experimental studies performed in adult animals have reported stimulation of neurogenic processes after brain ischemia across various model species. Neural stem cells located in both neuroproliferative regions (SGZ and SVZ) as well as in non-neurogenic regions (striatum and cerebral cortex) respond to different types of ischemia models (global, focal ischemia and intracerebral hemorrhagic stroke) by increasing proliferation and migration to areas of injury and generation of a variety of new functional cell types (Arvidsson et al., 2002; Nakatomi et al., 2002; Parent, 2003; Yamashita et al., 2006).

Accumulating evidence suggests that ischemic injury promotes extensive cell proliferation in the SGZ of the adult rodent brain. It was found that, depending on the insult severity, stem cell proliferation has been significantly upregulated around 1-2 weeks after the insult. According to the

reported data, roughly half of post-ischemic precursors acquire a neuronal phenotype in the granular cell layer of DG after global forebrain ischemia. Furthermore, newly generated granular neurons are able to extend dendrites into the molecular layer of DG establishing synapses with mature neurons born during development (Lledo et al., 2006; van Praag et al., 2002; Toni et al., 2008). Focal cerebral ischemia induced by MCAO in rodents also leads to enhancement of cell proliferation in the SVZ. According to the published reports new neurons continue to be added to the striatum for at least several months. However, the majority of newly-generated cells fail to survival and die between 2 and 5 weeks after ischemic stroke. Although the population of striatal neurons replaced by the neural stem cell progenies represented only small percent (about 0.2%) of the lost cells, this is proof that the brain is, to a limited extent, programmed to repair itself through the replacement of its cellular components (Marlier et al., 2015; Thored et al., 2006; Yamashita et al., 2006).

Although there have been many studies on ischemia–induced stimulation of neurogenesis in adults, much less work has been done to define such an effect in the immature brain subjected to hypoxic-ischemic insult.

The first evidence showing stimulation of neurogenesis after experimental neonatal ischemia has been provided by Plane et al. (2004), who demonstrated that acute HI brain injury in P10 mice stimulated cell proliferation and neurogenesis in the SVZ and peri-infarct striatum. This and following data (Hayashi et al., 2005; Ikeda et al., 2005; Yang and Levison, 2006) indicate that the SVZ maintains the capacity to promote cell proliferation in the damaged neonatal brain, and suggests that neural progenitors are a potential source for cellular repair after brain insults. Therefore, it was hypothesized that, compared to the adult, the neonatal mammalian forebrain maintains a similar or perhaps even greater potential for neuronal repair after HI injury. However, it is worth to point out that studies evaluating the proliferative capacity of the second neurogenic area – hippocampal SGZ, reported inconsistent data: either increased (Bartley et al., 2005) or reduced (Chang et al., 2006; Kadam et al., 2008) total counts of a new cells. These obvious discrepancies could be explained by differences in study paradigms and emphasize the importance of standardizing the experimental set-up.

It was expected that the HI-induced enhanced proliferation of neural progenitors will be followed by the migration of newly generated cells toward the injured brain areas, where they will acquire the desired phenotype during the differentiation process, enabling the damaged brain area to be reconstructed. However, the increase in post-HI proliferation of neural progenitors does not necessarily mean that all newborn cells will survive differentiation and maturation in an injured brain. As has been reported by Ikeda et al. (2005), only 1% of mature neurons and 4.6% of glial fibrillary acidic protein could be detected among new cells in the peri-infarcted area of the cortex and the striatum at 14 days after HI. So, it occurred that the endogenous repair processes do not

resolve the brain damage, as the capacity of endogenous regeneration proved to be rather limited and insufficient for replacing the loss neurons (Donega et al., 2013; Ong et al., 2005).

Thus, strategies are being sought to amplify the endogenous regenerative response by introducing molecules that might expand stem cell proliferation, maturation and survival.

1.3.5. Factors involved in the regulation of neurogenesis and regeneration

The mechanisms that will allow controlling neuronal fate are of great interest in the neurorepair field and represent a promising subject for the development of a factual clinical treatment. Because neurogenesis is a multi-stage process, involving a sequence of cells, regulation of neurogenesis is complex, even though the end results, the generation of none or fewer newborn neurons, might suggest otherwise. Adult neurogenesis seems to be very sensitive to a very broad range of stimuli and factors that alone or together affect the net result. Several extrinsic factors secreted by the stem cell niche, diffusing from the nearby vasculature or released from synaptic connections, relay the environmental influence onto the progression of progenitor proliferation and neuronal generation (Gould and Cameron, 1996; Palmer et al., 1999). However, the precise role of proteins involved in neurogenesis is still to be unraveled. It is in full agreement that neurogenesis is, to a large degree, quantitatively regulated through the control of new cell survival.

The key survival factors are several growth and neurotrophic factors, accepted as positive regulators of neurogenesis at early and postnatal development (Gould and Cameron, 1996; Palmer et al., 1999). A variety of them is upregulated following ischemic injury and may be involved in the ischemia-induced neuronal cell generation (Dempsey and Kalluri, 2007; Naylor et al., 2005; Yan et al., 2007) and by this may enhance brain recovery.

The next chapter provides a brief summary of the current knowledge concerning the role of neurotrophins and their potential contribution to neurogenesis and regeneration.

1.3.5.1. Neurotrophins

The neurotrophin family (NT) of neurotrophic factors is a family of closely related proteins that were first identified as survival factors for sympathetic and sensory neurons and have since been shown to control aspects of cell survival, development, and function of neurons in both the central and peripheral nervous system. The first neurotrophin discovered was NGF. Further work identified other members of the family such as BDNF, NT-3 and NT4/5. Two novel neurotrophins from the platyfish and carp have been cloned and designated as neurotrophin-6 and neurotrophin -7. These do not have orthologs in mammals or birds, however they appear to interact with the same receptors as mammalian proteins (Skaper, 2012). Neurotrophins are synthetized predominantly by neurons as precursors or pro-neurotrophins, which are cleaved to produce the mature proteins. The

mature proteins are about 12 kDA in size, form stable, non-covalent dimers, and are expressed at very low levels during development.

Classically, neurotrophins are thought of as neuronal survival factors that prevent the developmentally programmed death of peripheral neurons or the experimentally induced death in central neurons (Lewin and Barde, 1996). In the neonatal period neurotrophins and their receptors regulate neural cell survival in the developing nervous system. Moreover, they participate in the structural and functional regeneration of nerve tissue after ischemic damage (Holtzman et al., 1996; Schäbitz et al., 2000; Wu, 2005; Zhang et al., 2008). Local and parental administration of trophic factors (BDNF, IGF-1, GDNF, VEGF) helped to diminish the edema and size of brain lesion in focal ischemia (Hayashi et al., 1998; Kitagawa et al., 1998; Schäbitz et al., 2000; Wang et al., 2000). Several lines of evidence raise the possibility that these factors in conjunction with their receptors might be capable of regulating progenitor proliferation, migration, and/or differentiation. Conversely, pro-neurotrophins in many ways oppose the functions of mature proteins and induce cell death (Lu et al., 2005; Nykjaer et al., 2005; Woo et al., 2005).

The two most commonly investigated neurotrophins are Nerve Growth Factor and Brain Derived Neurotrophic Factor, which are discussed in the next part of this dissertation.

Nerve Growth Factor (NGF)

NGF was discovered in 1950 by Levi-Montalcini and Hamburger, as a trophic factor for sympathetic adrenergic and certain sensory neurons (Levi-Montalcini and Hamburger, 1951). At present, NGF emerges as a complex pleiotropic agent active on a surprisingly broad spectrum of cell populations and with biological functions within and outside of the nervous system (Skaper, 2017).

In the brain, relatively high NGF levels are found in the basal forebrain and regions innervated by the basal forebrain cholinergic neurons (hippocampus, cortex) where it serves a trophic function in cell development and maintenance. This protein is synthesized predominantly by neurons under physiological conditions (Thoenen, 1995). Studies *in vivo* and *in vitro* indicate that cerebral insults (e.g. local tissue injury, inflammation) upregulate NGF gene expression, mainly in astrocytes and microglia. Augmenting NGF supply to the target field effectively rescues some of the neurons that would otherwise have died. This remains in line with reports showing that intraventricular NGF administration prevents or significantly reduces severe neurological deficits following hypoxic-ischemic brain injuries. The overexpression of NGF in the brain increases the number of new neurons and neuronal precursors generated in the neurogenic areas, the SVZ and the SGZ. Interestingly, a recent report published by Han et al. (2017) showed that NGF improved viability, proliferation and differentiation of NSCs into functional neurons *in vitro*. Furthermore, NGF infusion into the cerebrospinal fluid (CSF) of the lateral brain ventricle results in a significant improvement in

motor and cognitive functions, with good recovery of level of awareness, purposeful movements and improvements in communicative skills. It is also suggested that NGF influences endothelial cell migration and angiogenesis after hypoxic–ischemic brain injuries and may act as an indirect activator of endothelial cell growth by stimulating the release of vascular endothelial growth factor (VEGF) as well as other vasoactive factors.

It should be, however, pointed out that this neurotrophin in conjunction with mast cells appears to be involved in neuroimmune interactions and tissue inflammation. This action of NGF, if left uncontrolled, could have deleterious effects on surrounding tissues and lead to chronic inflammatory processes. Thus, certain dysfunctions can occur not only as a result of neurotrophic factor deficiency, but also as a consequence of its excess.

Brain-Derived Neurotrophic Factor (BDNF)

BDNF, the endogenous agonist for the tropomyosin–related kinase B (TrkB) plays a key role in the CNS, promoting synaptic plasticity, neurogenesis, angiogenesis and cell survival (Lu et al., 2013a; Park and Poo, 2013; Rossi et al., 2006). BDNF, as well as its receptor TrkB, is broadly expressed in the developing and adult mammalian brain and contributes to diverse biological processes including differentiation, and facilitation of post-injury recovery and activity-dependent synaptic plasticity (Balaratnasingam and Janca, 2012; Bramham and Messaoudi, 2005). By binding to TrkB, it induces subsequent activation of downstream signaling cascades (the ERK1/2 and Akt pathways) to initiate the expression of specific genes responsible for proliferation, differentiation and cell survival. For example, in primary neuronal cultures BDNF protected hippocampal neurons against glutamate toxicity and glucose deprivation (Cheng and Mattson, 1994; Kokaia et al., 1994) and rescued cerebellar granular neurons from apoptotic stimuli (Leeds et al., 2005; Suzuki and Koike, 1997). It also prevented neuronal cell death *in vivo* when given by infusion or virus-mediated delivery (Almli et al., 2000; Schäbitz et al., 2007). There is evidence that BDNF treatment protects the brain against HI-induced damage, prevents loss of neurons and spatial memory dysfunction caused by the neonatal injury (Han and Holtzman, 2000; Han et al., 2000). Several published reports suggest that BDNF actions include anti-inflammatory and anti-apoptotic effects, promotion of neural regeneration, angiogenesis and contribution to cognitive functions and memory acquisition. However, it should be mentioned that despite of the established beneficial effect of BDNF there are multiple reasons that limit utilization of this factor as a practical therapeutic reagent, including its poor bioavailability, short plasma half-life, and a lack of specificity for neurotrophin receptors.

It should be noted that the protective effect of BDNF (promotion of survival, growth, and differentiation) is connected to the mature form of this factor, while pro-BDNF, when not serving as a precursor, is involved in neuronal death (Lee et al., 2001; Woo et al., 2005). It was found that pro-

BDNF facilitates LTD in the hippocampus, an effect that is directly opposed to the potentiating effect of mature BDNF through TrkB receptors (Lu et al., 2005). In another study, application of pro-BDNF to neuromuscular synapses *in vitro* decreased synaptic efficacy and caused retraction of presynaptic terminals by activation of p75.

1.3.5.2. Neurotrophin receptors

Biological effects of the mammalian neurotrophins are mediated through activation of two entirely distinct class of receptors - the three members of tyrosine receptor kinase Trk (TrkA, TrkB, and TrkC) and p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor superfamily. The signalling pathways activated by Trk and $p75^{NTR}$ receptors are presented on Figure 6.

The two receptor systems can function synergistically, antagonistically or independently of each other in different cell types (Kaplan and Miller, 1997; Lu et al., 2005). Interestingly, recent studies point to α9β1 integrin as a third receptor for NGF, BDNF, and NT-3 (Staniszewska et al., 2008).

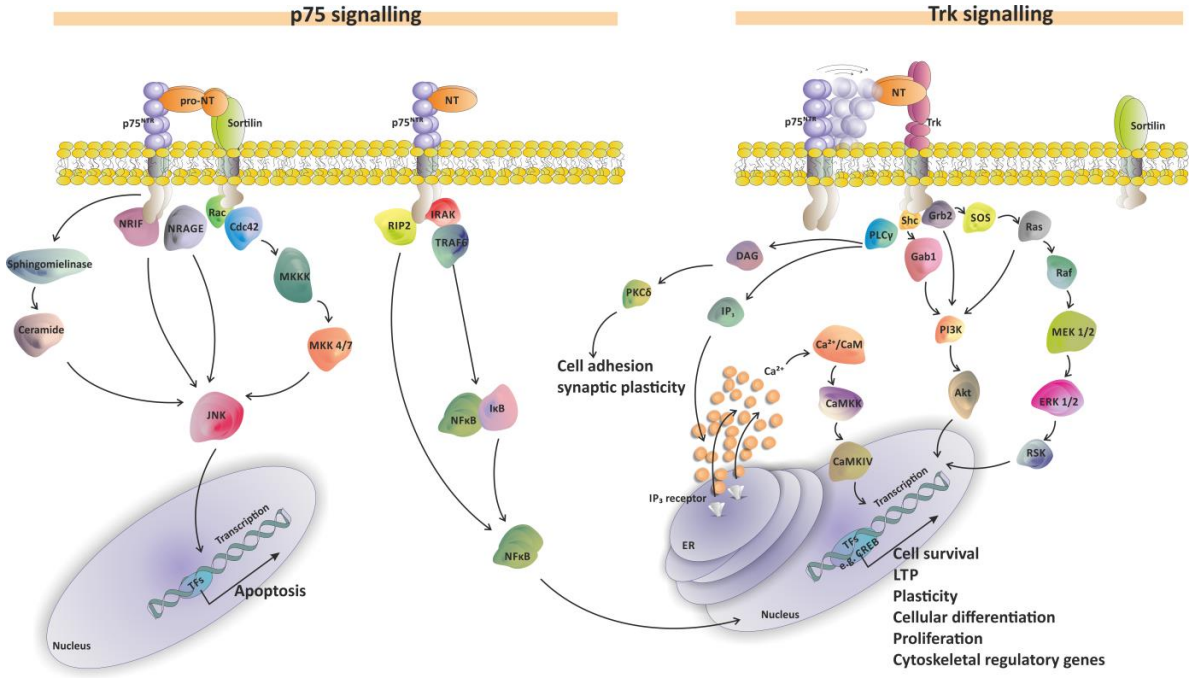


Fig.6. Signaling through neurotrophic receptors Trk and p75 (Nykjaer et al., 2004 - modified).

Tyrosine kinase receptors (Trk)

In mammals the Trk subfamily of tyrosine receptor kinases constitutes the second major class of neurotrophin receptors. Trk receptors are activated specifically by the mature and not precursors of the neurotrophin gene products. Thus, NGF binds to TrkA, BDNF and NT4/5 to TrkB, and NT-3 to TrkC. NT-3 can also interact, however with less efficiency, with TrkA and TrkB (Skaper, 2012).

The major site at which neurotrophins interact with Trk receptors is in the membrane proximal Ig-like domain. The neurotrophin binding to the receptor leads to dimerization and activation via transphosphorylation of the cytoplasmic kinase domain. The cytoplasmic domains of Trk receptors contain several tyrosine residues that phosphorylated may recruit a variety of adaptor proteins and enzymes that ultimately propagate the neurotrophin signal (Segal and Greenberg, 1996). The major pathways activated by Trk receptors are Ras-mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3 kinase (PI3K) and their downstream effectors. In addition, transfer of Trk receptors to different membrane compartments control the efficiency and duration of Trk-mediated signaling in part because many adaptor proteins are localized to specific membrane compartments (York et al., 2000). Activation of TrkA and TrkB receptors can also occur via a G-protein coupled receptor (GPCR) mechanism, in the absence of NGF and BDNF (Lee and Chao, 2001; Lee et al., 2002). The GPCR ligands – adenosine and pituitary adenylate cyclase activating peptide can activate Trk receptor activity to increase the survival of neuronal cells through stimulation of kinase B (Akt) activity. In contrast to neurotrophin treatment, Trk receptor activation by these factors is sensitive to transcriptional and translational inhibitors.

Trk receptor function is modulated by $p75^{NTR}$(described below) on several levels through promotion of ligand binding, accessibility to neurotrophins through axonal growth and retrograde transport to membrane compartments where internal engagement of neurotrophins with Trk receptors may promote signaling (Skaper, 2017).

Receptor $p75^{NTR}$

The p75 receptor is the first discovered member of the tumor necrosis factor receptor death-inducing superfamily. $P75^{NTR}$ is also referred to as CD271 in immune cells (Bothwell, 1995). While p75 transmebrane neurotrophin receptor does not contain a catalytic motif, it interacts with several proteins that relay signals important for regulating neuronal cell survival, differentiation, and synaptic plasticity. $P75^{NTR}$ was shown to bind each of the mature and pro-neurotrophin forms with approximately equal nanomolar affinity (Barker, 2004; Skaper, 2012). However, although the neurotrophins bind to p75 with similar affinity, each neurotrophin may exert different effects on cell function and viability. For example, the effect of NGF on oligodendrocyte cultures could not be reproduced by similar concentration of BDNF or NT-3.

Neurotrophin p75 receptor is widely expressed in the developing central and peripheral neurons during the period of synaptogenesis and developmental cell death (Davies, 2000). A subpopulation of peripheral and central glial cells, including radial glia and neural stem cells, also express p75 at various stages of development (Cragnolini et al., 2009). In the adult brain p75 is generally down-regulated, but increased again following different types of injury (Kokaia et al., 1998;

Lönngren et al., 2006). Although first identified for survival-promoting effects as a co-receptor for Trk, p75 is currently best known for its role in mediating neural cell death in the mature brain. A series of studies during the past decade have demonstrated that p75 signaling contributes to neuronal and glial cell damage (Casaccia-Bonnefil et al., 1996; Friedman, 2000), axonal degeneration and dysfunction during injury and cellular stress (Ibáñez and Simi, 2012). However, several examples of a role of p75 in promoting cell death during development have been uncovered (Haase et al., 2008; Raoul et al., 2000).

$P75^{NTR}$ contains a so-called death domain in the intracellular region, which mediates interaction with the cellular components, allowing the receptor to regulate intracellular signaling events (Dechant and Barde, 2002; Roux and Barker, 2002). Death signaling is mediated via one or more of the receptors' intracellular partners, resulting in c-jun kinase (JNK) activation, and subsequent p53, Bax-like proteins and caspase activation, (Coulson et al., 2004; Roux and Barker, 2002) as well as NFκB (Carter et al., 1996). In *in vitro* studies p75-linked signaling has also been found to regulate cell cycle progression and/or cellular maturation of different cell types, including neuroblasts (Lachyankar et al., 2003), embryonic striatal progenitors (Cattaneo and McKay, 1990), embryonic forebrain neurospheres (Hosomi et al., 2003), and subventricular zone cells (Gascon et al., 2005).

$P75^{NTR}$ signaling has also been shown to have some positive effects after injury, as observed in some cases of nerve remyelination (Song et al., 2006; Tomita et al., 2007) and EAE (Copray et al., 2004). The dual effect of p75 may result from intrinsic differences in the complement of intracellular mediators and co-receptors between developing and adult cells. On the other hand, different extracellular environment, such as the balance between neurotrophins and pro-neurotrophins, may also contribute to different, or even opposed p75 activities.

The p75 receptor provides a positive modulatory influence on TrkA function by increasing the number of high affinity binding sites (Mahadeo et al., 1994). Regulation of high affinity site formation by co-expression of TrkA and p75 provides an explanation for how these receptors may cooperate to increase neurotrophin responsiveness during development. Of note, the mechanism is not restricted to NGF, as BDNF signaling is also capable of interfering p75-mediated cell death through TrkB receptor (Davey and Davies, 1998). Given the opposite effect of TrkB and p75 activation on neurons, the molecular interventions for the specific activation of TrkB receptor, but not p75, is desired to protect neuronal injury. This remarkable observation indicates that p75 and Trk receptor can simultaneously influence life-and-death decisions depending on which ligands are available and whether Trk alone, Trk plus p75, or p75 alone are expressed.

2. RESEARCH AIMS AND OBJECTIVES

The neuroprotective/neurogenic effect of histone deacetylase inhibitors (e.g. VPA, TSA, SB) has been reported in several adult ischemia model studies; however this finding has received less attention in neonatal animals. Since many aspects of the evolving post-ischemic damage differ in the immature brain and thereby the efficacy of neuroprotection can differ between adults and neonates, extrapolating data obtained in the mature brain to neonates is generally unwise. Thus, the beneficial effect observed in adult stroke models prompted me to address the impact of histone deacetylase inhibition upon post-injury responses in the immature brain.

The main goal of this dissertation was to determine whether the application of Sodium Butyrate, a histone deacetylase inhibitor, will provide a neurogenic/neuroprotective action in a rat model of neonatal hypoxia-ischemia.

Experiments were conducted on an established, widely used in the world, model of hypoxic-ischemic brain injury, induced in 7-day old rats (Levine model adopted for young animals described by (Rice et al., 1981). All of the experiments were performed on animals divided into the following groups: Control (sham-operated), control (sham-operated) receiving treatment with SB and two HI groups – with and without treatment with SB. For the realization of the main objective the following detailed research aims were planned:

1) Effect of SB treatment on brain damage caused by HI.
2) Evaluation of SB treatment on cell proliferation and differentiation in two neurogenic areas: the SGZ of the hippocampus and SVZ of the lateral ventricles, at different time points after HI.
3) Evaluation of SB treatment on microglial/macrophage and astroglial response to neonatal hypoxia-ischemia.
4) Effect of SB treatment on the expression/activity of molecular mediators released by HI-injured brain cells, that may be crucial for the neurogenic response:

- Transcription factors (phospho-CREB, NF-κB, p53).
- Neurotrophic factors (brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF)) and their receptors (TrkB, p75).
- Kinases ERK1/2 and Akt, which are engaged in the neurotrophin signaling cascade.
- HSP70
- pro- and anti-apoptotic proteins (Caspase-3, Bax, Bcl-2).

5) Evaluation of functional outcome after HI injury and SB treatment using several behavioral tests (Open Field, Rotarod, Grip Test, Morris Water Maze, Ultrasonic Vocalizations).

3. MATERIALS AND METHODS

All animal experiments were conducted according to a protocol approved by the IV Local Ethics Committee for Animal Experimentation in Warsaw (83/2015). All efforts were made to minimize the number of animals and animal suffering in every step.

3.1. Experimental neonatal hypoxia-ischemia

Although an ideal HI model which fully represents the complex human pathophysiology is not available, Rice and Vanucci's 7-day-old rat procedure and extended to P7-9 mice has been the most commonly used rodent model for the study of asphyxia brain injury, since its first introduction in 1981 (Rice et al., 1981).

Cerebral hypoxia-ischemia was induced in seven-day-old (P7) Wistar rats of either sex by a permanent unilateral common carotid artery ligation, followed by systemic hypoxia (Rice et al., 1981; Vannucci and Vannucci, 1997). As was reported the ligation alone does not decrease cerebral perfusion below critical levels and the addition of hypoxia is required to cause brain infarct (Vannucci and Vannucci, 2005). Pups were anesthetized with isoflurane (AbbVie Polska) (4% induction, 2.0% maintenance) carried by O_2. Once they were fully anesthetized, a midline neck incision was made and the left common carotid artery was isolated, double ligated with surgical silk and cut between two ligatures. The incision was then sutured with monofilament nylon. Sham-operated animals underwent the same surgical procedure without the ligation of the carotid artery. The duration of anesthesia lasted on average 5 min. After the surgical procedure, the rat pups were returned to their home cage for 1 hour recovery. Hypoxia was induced by placing the animals in a preheated to 35°C chamber and exposing them to a gas mixture of 7.6% oxygen in nitrogen for 1 hour. After the hypoxic exposure the pups were returned to their dams. They were reared at 20°C environmental temperature with a light:dark cycle of 12:12 hours and food and water ad libitum.

The undamaged hypoxic hemisphere, as well as age-matched sham-operated animals served as controls. Pups from each litter were randomly assigned to 4 experimental groups (5 rats per group and time point): 1) control group (vehicle treatment) 2) control animals (SB treatment) 3) animals which underwent HI (vehicle treatment), 4) animals which underwent HI (SB treatment). Animals were sacrificed at specific time points (1, 2, 3, 6, 7, 9, 11, 14 or 28 days) after the injury.

The procedure has several advantages, including relatively simple methodology, reproducible low percent of mortality and its suitability for long-term behavioral and neurological experiments. In addition, the 7-day-old rat brain was found to have equivalent brain maturity to that of a full-term human infant (Goodman and Tessier-Lavigne, 1998). A limitation of this model is animal to animal variability of injury (Mallard and Vexler, 2015). Other animal models were also suggested in different

animal species, such as non-human primates, newborn sheep or lamb. These models have critical drawbacks including the expense and difficulty in the use of large animals, absence of clinical evidence for brain injury and consequently unavailability of neurobehavioral scoring (Northington, 2006).

It is worth to underline, that studies investigating HI-mediated neuronal damage have utilized the contralateral hemisphere as an "internal control" However, unilateral ischemia has been shown to decrease contralateral regional cerebral blood flow and metabolism (Dobkin et al., 1989; Heiss et al., 1993; Lagrèze et al., 1987). In addition to contralateral electrophysiological changes, small alterations in gene expression, inflammatory markers, and neuronal density have been documented following HI in the contralateral hemisphere (Gilby et al., 1997; Towfighi et al., 1995; van den Tweel et al., 2006) and it is now evident that inflammatory processes are central to CNS injury following HI. All these suggest that the contralateral hemisphere may not be a valid control in procedures of HI, therefore the present study also engaged control, sham-operated animals to receive credible results.

3.2. Drug administration and bromodeoxyuridine labeling

Drug treatment Rats subjected to HI or sham-operated were treated with subcutaneous injections of sodium butyrate (SB, Sigma-Aldrich; in a dose of 300 mg/kg body weight) according to the published data (Kim et al., 2007a, 2009), or vehicle (saline), starting immediately after hypoxic exposure and lasting 5 consecutive days.

Bromodeoxyuridine labeling Endogenous cell proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) cell–incorporation. BrdU (Sigma-Aldrich) dissolved in sterile physiological saline (0.9% NaCl plus 0.007 N NaOH) was administered intraperitoneally (50 mg/kg body weight per injection). Two-injection paradigms were used. In the first paradigm animals received a single dose of BrdU and were sacrificed 24 hours after the injection. This procedure was used to determine the number of cells that incorporated BrdU during a 24 hour period at a specific time point after HI (3, 6, 9, 11, 14 days). In other experiments the animals received BrdU injections twice daily (12 hours apart) for 3 consecutive days starting 4 days after the onset of hypoxia-ischemia. Animals in this group were sacrificed 14 and 28 days after the insult. This allowed determining the phenotype of newborn cells

3.3. Brain injury evaluation

Fourteen days after the insult (at postnatal day 21) the pups were anesthetized with 100 mg/kg body weight ketamine (Vetoquinol) combined with 10 mg/kg body weight xylazine

(Vetoquinol), decapitated and the brains were dissected. The brain stem and cerebellum were removed from the forebrain. The two cerebral hemispheres were separated in the midline and weighed on the high precision balance. The brain damage was assessed by the weight deficit (in %) of the ipsilateral (injured hypoxic-ischemic) hemisphere relative to the contralateral (hypoxic only) hemisphere (Andiné et al., 1990).

Brain damage was also evaluated neuropathologically by Hematoxylin-Eosin (HE) staining. On P13 and P21 (6 and 14 days after HI, respectively) the pups were anesthetized with 100 mg/kg body weight ketamine combined with 10 mg/kg body weight xylazine and perfused with a fixative solution (4% paraformaldehyde, PFA [MP Biomedicals], in 0.1 M phosphate buffer, PBS [Gibco] pH=7.4). The brains were removed and submerged in the same fixative solution for 4 hours at 4°C. Following postfixation brains were cryoprotected overnight in 30% sucrose (MP Biomedicals) solution (in 0.1 M PBS), frozen rapidly using dry ice and placed in -80°C storage.

Prior to cutting, frozen brains were moved to the cryostat, brought to -20°C and coronal cryostat sections (20 µm) were mounted on slides coated with 2% silan solution (Sigma-Aldrich) in acetone (Chempur). Tissue sections were stained in Hematoxylin (LEICA) for 6 minutes and then washed in running tap water for 30 min and stained in 1% Eosin Y (POCH) for 2 minutes. After this step, the slides were dehydrated in increasing concentration of ethanol alcohols (POCH; 70%, 96%, 100%, each 3 min) and cleared in xylene (Chempur, 2x3 min). Finally they were mounted in mounting media (DePeX mounting medium, Gurr), coverslipped and examined using light microscopy.

3.4. Tissue preparation

At the scheduled time points (3, 6, 9, 11, 14 and 28 days after HI), anesthetized animals were perfused transcardially as described in “Brain injury evaluation” section.

For biochemical analysis animals were sacrificed (1, 2, 3 and 7 days after HI) through decapitation and the whole hemispheres were frozen on dry ice. All tissue samples were stored at -80°C until used.

3.5. Immunohistochemistry

Prior to cutting, frozen brains were moved to the cryostat and brought to -20°C. Next they were cut (30 µm thick) at the level of the dorsal hippocampus or lateral ventricles in serial order to create 10 series sections. Free floating sections were kept in an anti-freeze buffer (0.01 M NaH_2PO_4, 0.04 M Na_2HPO_4, 30% ethylene glycol, 30% glycerin) at -20°C. Double fluorescent immunohistochemistry was performed after rinsing the sections in PBS (3x5 min) in room temperature (RT).

For detection of BrdU incorporation, DNA was first denaturated by incubation of sections with 2N HCl (Chempur) at 37°C for 1 hour, and rinsed for 15 min in 0.1 M sodium tetraborate (0.2 M H_3BO_3, 0.05 M $Na_2B_4O_7x10H_2O$, pH 8.4) at RT. After blocking with 10% normal goat/donkey serum (Sigma-Aldrich) in PBS containing 0.25% Triton X-100 (Sigma-Aldrich) for 60 min and washing with PBS (3x5 min) at RT, sections were incubated with anti-BrdU overnight at 4°C. Following the washing procedure (3x5 min PBS, RT), the primary antibodies were revealed by appropriate secondary FITC-conjugated antibodies 60 min at room temperature and in the dark.

Differentiation of BrdU-positive cells was monitored with markers labeling neurons and oligodendrocytes at various stages of maturation (DCX, calbindin, NeuN, NG2, O4), as well as microglia (ED1, Arg-1, IL-1β) and astrocyte (GFAP) markers. After BrdU staining, the brain-tissue sections were rinsed in PBS (3x5 min, RT) and incubated with primary antibodies overnight at 4°C. The following day the sections were washed in PBS (3x5 min, RT) and exposed to secondary appropriate Cy3-conjugated secondary antibodies for 1 hour at room temperature. Subsequently, tissue sections were rinsed in PBS (3x5 min, RT) and nuclei were labeled with the fluorescent dye Hoechst 33258 (2 μg/ml PBS; Invitrogen) by incubating the sections for 30 min at RT. After final washing in PBS (3x5 min, RT) sections were mounted on slides and coverslipped using DAKO fluorescent mounting medium (Agilent). In all cases negative controls were processed in the same manner on adjacent sections but with the primary antibodies omitted. All of the above procedures were done in the dark. Primary and secondary antibodies are listed in Table 4 and 5.

Phenotype	Antibody (host)	Company	Dilution (isotype)
Proliferating cells	Polyclonal anti-BrdU (sheep)	***Abcam***	1:500 (IgG)
Proliferating cells	Monoclonal anti-BrdU (mouse)	***Santa Cruz***	1:100 (IgG1)
Neuroblasts	Polyclonal anti-Doublecortin *DCX* (rabbit)	***Cell Signaling***	1:200 (IgG)
Mature granule neurons	Monoclonal anti-Calbindin (rabbit)	***Cell Signaling***	1:200 (IgG)
Mature neurons	Monoclonal anti-NeuN (mouse)	***Millipore***	1:200 (IgG)
Oligodendrocyte progenitors	Polyclonal anti-NG2 (rabbit)	***Millipore***	1:200 (IgG)
Non-myelinating oligodendrocytes	Monoclonal anti-O4 (mouse)	***Millipore***	1:200 (IgM)
Activated microglial/macrophage cells	Monoclonal anti-ED1/CD68 (mouse)	***AbD Serotec***	1:100 (IgG1)
M2 microglia marker– Arginase -1 (Arg-1)	Polyclonal anti-Arg-1 (goat)	***Santa Cruz***	1:250 (IgG)
M1 microglia marker – IL-1β	Polyclonal anti-IL-1β (rabbit)	***Santa Cruz***	1:250 (IgG)
Astrocytes	Polyclonal anti-BrdU (rabbit)	***DAKO***	1:200 (IgG)

Table 4. List of primary antibodies used for immunohistochemistry.

Antibody	Fluorophore	Company	Dilution (isotype)
Anti-sheep	Alexa Fluor 488	***Invitrogen***	1:500 (IgG)
Anti-mouse	Alexa Fluor 546		1:500 (IgG)
Anti-mouse	Alexa Fluor 488		1:500 (IgG1)
Anti-mouse	Alexa Fluor 546		1:500 (IgG1)
Anti-mouse	Alexa Fluor 546		1:500 (IgGM)
Anti-rabbit	Alexa Fluor 488		1:500 (IgG)
Anti-rabbit	Alexa Fluor 546		1:500 (IgG)
Anti-goat	Alexa Fluor 488		1:500 (IgG)

Table 5. List of secondary antibodies used for immunohistochemistry.

3.6. Microscopy visualization

Double labeling determining the expression of lineage markers by BrdU-positive cells was verified using a confocal laser scanning microscope (LSM 780, Carl Zeiss, Germany) or for images obtained by tile scan function using a Spinning Disk confocal microscope (Cell Observer SD, Carl Zeiss, Germany) applying a 10x or 20x objective and crop function with ZEN software. A helium-neon laser (543 nm) was utilized in the excitation of Alexa Fluor 546 (Cy3), an argon laser (488 nm) was applied in the excitation of FITC and a diode laser (405 nm) was used for visualization of Hoechst. The number of BrdU-positive cells in the entire DG area was assessed on an average of five hippocampal sections per animal. To avoid double counting, adjacent sections were not analyzed. Every section was evaluated using a computerized system, and the positive cells were displayed on a computer screen. All of the counting was performed using ImageJ 1.46 software. The number of positively labeled cells as well as fluorescence intensity was assessed in an area of 1,44 mm^2.

3.7. Western Blot analysis

3.7.1. Preparation of protein extracts

Brain tissue was homogenized in RIPA lysis buffer (10 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 1% Triton X-100, PMSF 0.1 mg/ml) and a proteinase and phosphatase inhibitors cocktail (Life Technologies, 1:100). Lysates were clarified by centrifugation at 13000 x g for 10 min at 4°C. Next, the supernatants and cell pellets (resuspended in RIPA buffer) were collected and protein concentrations were measured as described in section ***Protein concentration measurement*** using Lowry assay. Afterwards, protein samples were denatured for 5 minutes in 100°C with an addition of 2x Laemmli buffer (62.5 mM Tris-HCl o pH 6.8; 10% glycerol, 2% SDS, 1.5% β-mercaptoethanol, 0.05% bromophenol blue) in a 1:2 volume ratio.

3.7.2. Protein concentration measurement

Protein concentrations were determined with a Bio-Rad DC™ protein assay kit using 96-well plates. The calibration curve was created using serial concentrations (10, 15 and 20 µg/ml) of Bovine Serum Albumin (BSA; Sigma-Aldrich) diluted in deionized water. Deionized water was also used as blank. The final reaction solution (460 µl) contained: 2 µl protein, 8 µl water, 50 µl reagent A (Bio-Rad, #500-0113) and 400 µl reagent B (Bio-Rad, #500-0114). After 15 minutes of incubation in room temperature the absorbance at 450 nm wavelength in every sample was measured in triplicates using a spectrophotometric plate reader Fluorostar Omega (BMG LabTech).

3.7.3. SDS-PAGE and Western blotting techniques

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). A double layer of polyacrylamide gel (10 cm x 5 cm x 0.75 mm) was prepared manually. Polymerization of the bottom resolving gel (7.5, 10 or 12% acrylamide/bis-acrylamide (9:1), 375 mM Tris-HCl (pH=8.80), 0.1% SDS, 0.005% ammonium persulfate) and the upper stacking gel (4% acrylamide/bis-acrylamide (9:1), 125 mM Tris-HCl (pH=6.80), 0.1% SDS, 0.05% ammonium persulfate) was induced by the addition of TEMED at a final concentration of 0.05%. The gel was placed in a Mini Protean (Bio-Rad) electrophoresis chamber filled with Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 0.5% SDS). Pellet and supernatant samples containing 50 or 100 µg of protein and molecular weight size markers were loaded on the gel and separated at a 80V through the stacking gel and 120 V through the resolving gel.

3.7.4. Electroblotting

Proteins separated by electrophoresis were transferred onto nitrocellulose membranes (0.45 µm Hybond-Extra C, Amersham) in a chamber filled with transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) at a constant voltage of 100V and amperage not higher than 300 mA for 60 minutes at 4^{o}C. To estimate the transfer efficiency, the membrane was stained with Ponceau S Solution (Sigma; 1% in 0.1% acetic acid) and the gels were stained with Coomassie blue (Fluka). Colored membranes were washed with distilled water and proteins were analyzed immunochemically.

3.7.5. Protein immunodetection

Protein immunodetection was carried out using specific primary antibodies (according to the protocols provided by manufacturer; Table 6), and then with secondary antibodies conjugated with horseradish peroxidase (HRP) (Table 7). In order to avoid non-specific binding of antibodies the nitrocelluloses were incubated in a Western Blot blocking buffer containing a 5% solution of non-fat

milk in TBST (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0,05% Tween 20) or a 5% solution of bovine albumin in TBST for 1 hour at room temperature. The blocked membranes were incubated overnight at 4°C with primary antibodies suspended in TBST. Afterwards, the membranes were washed 3x5 min with TBST and incubated for 1 hour at RT with appropriate HRP-conjugated secondary antobodies in WB blocking buffer. Further the membranes were washed 3 times for 5 min in TBST and incubated for 1 min in an ECL chemiluminescence kit (Amersham). The chemiluminescent reaction was detected by membrane exposition to a X-ray Hyperfilm™ ECL (Amersham) film.

To verify specific reactions the same procedure was used with the omission of the primary antibody and to verify an equal loading of protein per line the beta-actin antibody was used as an internal control for each immunoblotting.

Semi-quantitative evaluation of protein levels detected by immunoblotting was performed by computer assisted densitometric scanning (LKB Utrascan XL, Program GelScan). The level of protein immunoreactivity was determined by frequent analysis of multiple immunoblots.

Antibody (host)	Company	Dilution
Monoclonal anti-βactin (mouse)	MP Biomedicals	1:500
Polyclonal anti-Bcl-2 (rabbit)	Cell Signaling	1:1000
Polyclonal anti-Bax (rabbit)	Cell signaling	1:1000
Polyclonal anti-acetylH3 (rabbit)	Millipore	1:1000
Monoclonal anti-pro-BDNF (mouse)	Santa Cruz	1:1000
Polyclonal anti-TrkB (rabbit)	Santa Cruz	1:200
Polyclonal anti-phospho-TrkB (pTyr 515) (rabbit)	Thermo Fisher	1:200
Monoclonal anti-p75 (rabbit)	Cell signaling	1:500
Polyclonal anti-ERK 1/2 (rabbit)	Cell Signaling	1:1000
Monoclonal anti-phospho-ERK 1/2 (Thr202/Tyr204) (mouse)	Cell Signaling	1:1000
Polyclonal anti-Akt (rabbit)	Cell Signaling	1:1000
Polyclonal anti-phospho-Akt (Ser473) (rabbit)	Cell Signaling	1:1000
Monoclonal anti-phospho-CREB (Ser133) (mouse)	Millipore	1:500
Monoclonal anti-NfκB p65 (mouse)	Cell Signaling	1:1000
Polyclonal anti-HSP70 (rabbit)	Cell Signaling	1:1000
Polyclonal anti-p53 (rabbit)	Cell Signaling	1:1000

Table 6. List of primary antibodies used for Western Blot.

Antibody	Company	Dilution
Anti-mouse	Sigma-Aldrich	1:4000
Anti-rabbit	Sigma-Aldrich	1:8000

Table 7. List of secondary antibodies used for Western Blot.

3.8. Quantitative measurement of BDNF and NGF protein concentration

To estimate the amount of BDNF and NGF in lysates prepared from both brain hemispheres, the ChemiKine Brain Derived Neurotrophic Factor, Sandwich ELISA (Millipore) and the ChemiKine Nerve Growth Factor, Sandwich ELISA (Millipore) test was applied according to the supplier's instructions. After performing the Sandwich ELISA assays, the plates were read at 450 nm using a spectrophotometric plate reader Fluorostar Omega (BMG LabTech). A standard curve was plotted for each plate. BDNF and NGF concentrations were assayed from the regression line of the BDNF and NGF standards.

3.9. Quantitative measurement of caspase-3 activity

To estimate the level of activated caspase-3 in lysates obtained from both brain hemispheres, the Caspase-3 Fluorescence Assay Kit (Cayman Chemical) was applied according to the supplier's instructions. First, hemispheres were homogenized in 1 mL of a buffer containing 25 mM HEPES, 250 mM Sucrose and 1 mM EDTA and protein concentration was measured as described in section ***Protein concentration measurement***. Next 100 µl of Cell-Based Assay Lysis Buffer (included in the Kit) was added to 100 µl of each homogenate and was incubated with shaking for 30 min in RT. The samples were then centrifuged at 800 x g for 10 min and the acquired supernatants were used for further analysis. Briefly, the kit employs a specific caspase-3 substrate, N-Ac-DEVD-N'-MC-R110, which upon cleavage by active caspase-3 generates a highly fluorescent product that is easily quantified. The fluorescence intensity of each well was read using a spectrophotometric plate reader Fluorostar Omega (BMG LabTech; excitation = 485 nm, emission = 535 nm). Caspase 3 activity expressed in relative fluorescence unit (RFU) was normalized against protein concentration.

3.10. Quantitative polymerase chain reaction (real-time PCR)

Gene expression of neurotrophins (BDNF and NGF) was evaluated in the brain hemispheres obtained from rats 72 hours and 7 days after HI. Total RNA was isolated by homogenizing 50-100 mg of tissue in 1 ml of TRIzol Reagent (Life Technologies) and incubating the homogenate 5 min in RT. Next, 200 µl of chloroform (Sigma-Aldrich) was added and the samples were mixed by shaking. After 2-3 min of incubation at RT, samples were centrifuged at 12000 x g for 15 min at 4°C. Following

centrifugation, the mixture separates into lower, phenol/chloroform phase, an interphase, and an upper aqueous phase. RNA remains exclusively in the aqueous phase and this phase was transferred carefully without disturbing the interphase into a fresh tube and 500 µl of isopropanol (Sigma-Aldrich) was added. After mixing and incubating the samples for 10 min in RT, they were centrifuged at 12000 x g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol (1 ml of 75% ethanol per 1 ml of TRIZOL). The samples were mixed and centrifuged at 7500 x g for 5 min at 4°C. Thereafter the supernatant was removed, the RNA pellet was air-dried and dissolved in 150 µl of DEPC-treated water (Ambion) and incubated 10 min in 58°C. The quality and concentration of RNA was verified by spectrophotometry with the Nanodrop™ apparatus. The samples containing 1 µg of total RNA were reversely transcripted using High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR analyses of cDNA samples (300 ng) with designed specific primers (Table 8) and Fast SYBR Green Master Mix (Applied Biosystems) were performed in 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction parameters were as follows: (1) holding stage, 20 s at 95°C; (2) cycling stage (40×), 3 s at 95°C and 30 s at 60°C; and (3) melt curve stage, 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C. Each sample was tested in triplicate during two analyzing sessions. The fluorescence signal from specific transcript was normalized against that of reference gene (SDHA), and threshold cycle values (ΔCt) were quantified as fold changes by the $2^{-\Delta\Delta CT}$ method.

Gene	Forward primer sequence	Reverse primer sequence
BDNF	5'-CGGCTGGTGCAGGAAAGCAA-3'	5'-TCAGGTCACACCTGGGGCTG-3'
NGF	5'-CCCGAATCCTGTAGAGAGTGG-3'	5'-GACAAAGGTGTGAGTCGTGG-3'
SDHA	5'-CCCTGAGCATTGCAGAATC-3'	5'-CATTTGCCTTAATCGGAGGA-3'

Table 8. List of designed primers used in reverse transcription and quantitative real-time RT-PCR analysis.

3.11. Behavioral tests

The studies were performed in Behavior and Metabolism Research Laboratory, Mossakowski Medical Research Centre, Warsaw in collaboration with dr hab. Robert Kuba Filipkowski and mgr inż. Rafał Polowy. Experimental animals were divided into 3 groups: control (C), n=12; hypoxia-ischemia (HI), n=11; and hypoxia-ischemia with sodium butyrate treatment (HI+SB), n=12. During behavioral experiments, rats were kept in a 12 hour light-dark cycle with water and food provided *ad libitum*. The experiments were done during the light phase of the cycle. Animal behavior was monitored at

postnatal day 33-83 (P33-83). All testing and training was conducted by an observer blind to the treatment group in a sound attenuated room.

3.11.1. Open Field

Open Field (OF) was initiated at P33-34 and performed for 3 days. OF box dimensions were 55 x 55 x 50 cm. Rats were gently placed in the middle of the OF floor. Recording lasted for 15 min. After each trial the apparatus was cleaned with 10% ethanol solution. Animal behavior was recorded with Basler acA1300-60 GigE camera (Bassler AG, Germany) and scored using Ethovision XT 10 (Noldus Information Technology, Netherlands). For the analysis, OF floor was virtually divided into three zones: i. border, 9.2 cm wide; ii. middle, 9.2 cm wide and iii. center square, 18.3 x 18.3 cm. The following parameters were measured: latency to the first entrance to a zone, frequency of entering zones, percent time duration in each zone, mean velocity, and total distance moved (compare (Ben Abdallah et al., 2013; Jedynak et al., 2012; Kiryk et al., 2008).

3.11.2. Rotarod

Rotarod (Accelerating Rota-Rod 7750,TSE systems, Germany) was started at P39-40 and conducted according to (Karalis et al., 2011). On the first day only, the test was preceded by habituation, i.e. placing the rat on a stationary cylinder for 30 s and thereafter for 2 min with a constant low speed rotation (4 rpm). Animals that fell from the rod were placed again on it until they were able to stay for 60 s. After at least 10 min rest, the animals were tested in accelerating conditions. The cylinder accelerated from 4 rpm to 40 rpm in 300 s. The time of the trial was scored when the rat fell from the cylinder, spun with the cylinder 3 times consecutively without walking or reached a maximum of 500 s without falling. The device was cleaned with 10% ethanol solution between animals. Each rat was scored once daily for 4 days.

3.11.3. Grip test

Grip test (Bioseb BP, In Vivo research Instruments, France) was initiated at P46-47 and done for two consecutive days (Simard et al., 2012; Steiner et al., 2011). To measure the forepaw grip strength of the rat, it was held by the trunk and the base of the tail. Then it was guided onto a metal grid 90.5 cm square opening) attached to a force transducer and encouraged to grab It for forepaws only. Then the animal was steadily pulled backwards until it lost hold of the grid. Three measurements per rat were taken with at least 1 min interval between trials to let the animal rest. The grip strength was expressed in newtons.

3.11.4. Morris Water Maze

Morris Water Maze (MWM; Garthe et al., 2014; Jaholkowski et al., 2009; Kiryk et al., 2011) was started at P62. The pool was 150 cm in diameter, the water temperature was of around 25°C, and it was dyed grey to discourage the animals from diving. A square platform (10 x 10 cm) was submerged 1.5 cm underneath the surface in the middle of one of the quadrants. Rats home cages were left in the pool room for at least 1.5 hour to accommodate. For each trial, the rat was placed on the platform for 60 s, and then it was put, facing the walls of the pool, into the water at one of 4, random-chosen, starting positions and had 60 s to find the platform. In case of failure, the animal was guided to the platform where it stayed for 60 s. There were 2 trials per day for 6 days. On the seventh day the probe trial was performed with the platform removed and the rats placed in the opposite quadrant. The trial lasted for 60 s; it was repeated a week later. Animal behavior was recorded and scored as in OF experiments. The Visible Platform Test was performed in the same conditions but the tank was covered around with curtains to remove visual cues of the room. The platform was marked with a flag and placed consecutively in 4 different positions. The rat was put into the water in the opposite side of the pool related to the platform. Time required for the animal to reach the platform was scored.

3.11.5. Ultrasonic vocalizations

Ultrasonic vocalizations (USV) were evoked by tickling. The procedure was initiated at P81 with some of the above mentioned animals (C, n=6; HI, n=6; HI+SB, n=5). It consisted of two days of pre-training with a familiar experimenter and the test day. The procedure was the same during pre-training and the experiment. Namely, the rat was placed in the tickling cage (57 x 38 x 20 cm) with a high sensitivity condenser microphone hung 30 cm above the center of the cage floor. The rat waited for 30 s in the tickling cage before the procedure. Then it was tickled for 15 s firstly by rapid finger movement around the neck area and the by flipping the animal on its back and rapidly moving fingers on its belly. The next 15 s were spent by moving the experimenters hand around the cage and allowing the rat to chase it. This was repeated 4 times (Panksepp and Burgdorf, 2000). USV were automatically scored on the spectrogram (Hamming, frame size 100%, overlap 50%) with Avisoft SASLab Pro software.

3.12. Statistical Analysis

GraphPad PRISM 5.0 software was used for the statistical analysis of the received data (excluding behavioral test results). Comparisons between animal groups were performed using one-way analysis of variance (ANOVA) with post-hoc Bonferroni test for multiple comparisons. All values were expressed as mean ±SD. All data received from behavioral tests are represented as means with

standard error of the means (SEM). The effects of behavioral experiments were analyzed with ANOVAs. Significant main effects or interactions were followed up with post-hoc analysis (Duncan), where appropriate. The calculations were made using STATISTICA 7.1. (StatSoft Inc., 2005).

4. RESULTS

4.1. Effects of sodium butyrate on brain damage after neonatal hypoxia-ischemia

All pups exposed to hypoxia-ischemia were lesioned in the left (hypoxic-ischemic) hemisphere. Dorsal view of the rat brain 2 weeks after the insult presents damage (atrophy) of the ipsilateral (hypoxic-ischemic) hemisphere and brain asymmetry. It should be noted that this model of HI leads to different degree of damage reflecting individual response to the insult (please compare Fig. 7A upper panel). After sodium butyrate (SB) treatment none of the HI hemispheres showed atrophy. However, in a few cases low degree of brain asymmetry could still be visible (Fig. 7A lower panel left image).

In order to evaluate the effect of sodium butyrate on brain damage caused by neonatal hypoxia-ischemia, the wet weight of ipsilateral hemispheres was compared to the contralateral ones. The results are presented on Fig. 7B. Two weeks after hypoxia ischemia the mean weight deficit of the ipsilateral (injured) hemisphere amounted 49.81% and it decreased to 27.05% after SB treatment. In the same conditions the weight of the intact contralateral side was not different from the respective sham control.

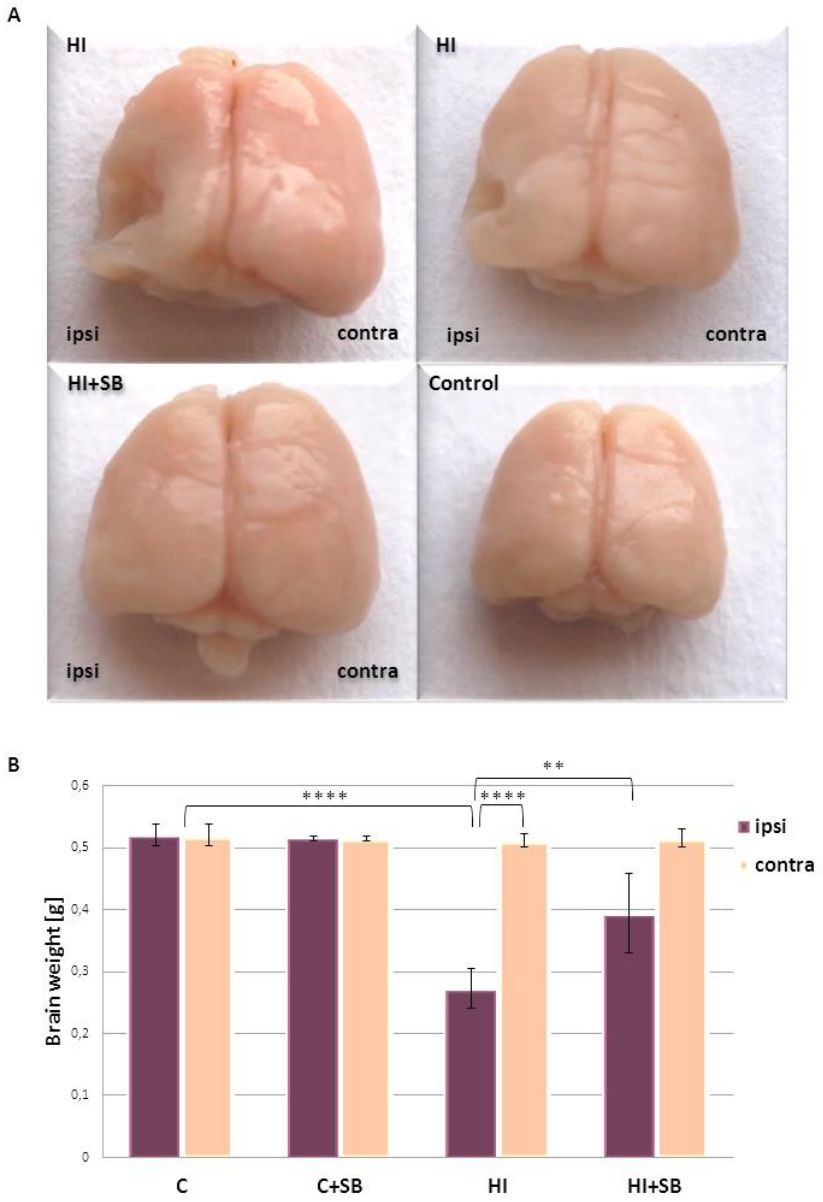


Fig.7. Effect of sodium butyrate on rat brain damage after neonatal hypoxia-ischemia. The degree of damage was demonstrated as the weight deficit of the ipsilateral (injured hemisphere) compared to the contralateral one. (A) Dorsal view of rat brains after neonatal HI with and without SB treatment. (B) Weight (g) of the ipsilateral and contralateral hemispheres in rats with or without SB treatment. The values are means ± SD of 5 animals in each experimental group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, **p < 0.01 and ****p < 0.0001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

Both the left and right brain hemispheres of all rats (sham control, HI with or without SB treatment) were subjected to histological evaluation at 6 and 14 days after the insult. At the earlier time point, coronal sections (cut at the level of the lateral ventricles as well as hippocampus) stained with hematoxylin and eosin show a massive loss of neurons and signs of cerebral edema with swollen

cells throughout the ipsilateral frontal cortex exclusively (Fig. 8A). Two weeks after HI brain damage evolved, leading to ventricular enlargement and in some cases to loss of the hippocampus (Fig. 8B). These brains however, were not considered in further immunohistochemical studies. At both time points administration of SB immediately after HI provided almost complete neuroprotection in comparison with non-treated animals. Neither neuronal loss nor edema was observed. Furthermore, the brain slices demonstrated proper cytoarchitecture.

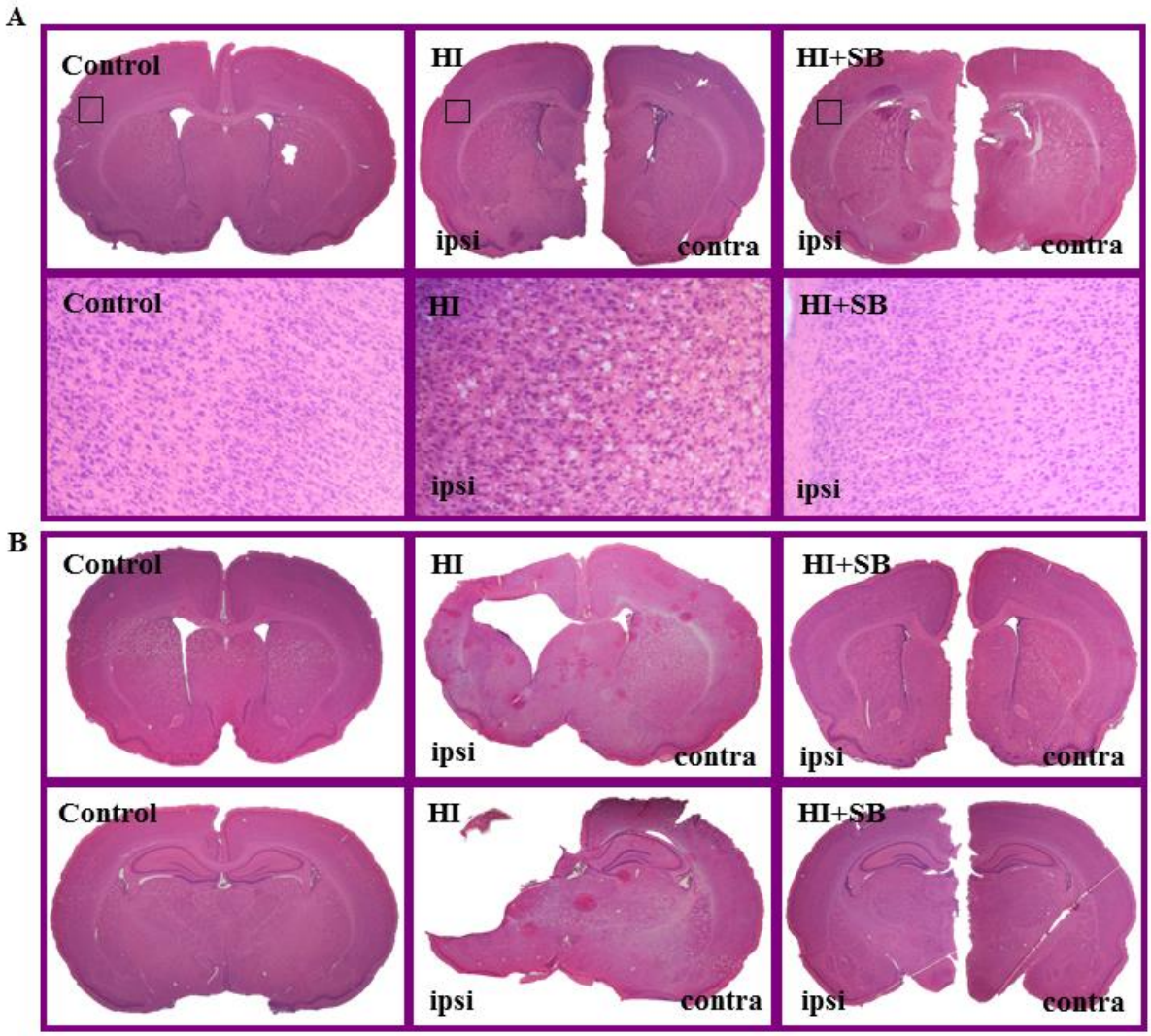


Fig.8. Effect of sodium butyrate on hypoxia-ischemia-induced brain damage. Brain coronal sections from sham control animals and from animals 6 (A) and 14 days (B) after HI (with or without SB treatment) stained with hematoxylin-eosin (HE). (A) Lower panel represents magnification (100×) of the ipsilateral hemisphere (area marked with rectangles in upper panel). (B) Lower panel shows extremely massive damage. Photomicrographs are representative of observations made from five animals per group. Abbreviations: ipsi *ipsilateral*, contra *contralateral*

4.2. The effect of sodium butyrate on the acetylation of histone 3

At first the level of acetylated histone 3 (Acetyl-H3) in rat brains after the application of 1, 3, or 5 doses of sodium butyrate was measured. Figure 3 shows respective representative immunoblots probed with antibody specific to acetylated histone H3 (Fig. 9A) together with the densitometric analysis (Fig. 9B). As depicted, the injection of SB resulted in significant increase of Acetyl-H3 immunoreactivity by 31% over vehicle control, but only in sham operated animals at postnatal day 10. In contrast, no statistically significant changes in Acetyl-H3 level were noted in the brains after HI insult, however a noticeable tendency of histone acetylation to decrease was observed in the ipsilateral hemisphere at 72 hours of recovery (P10).

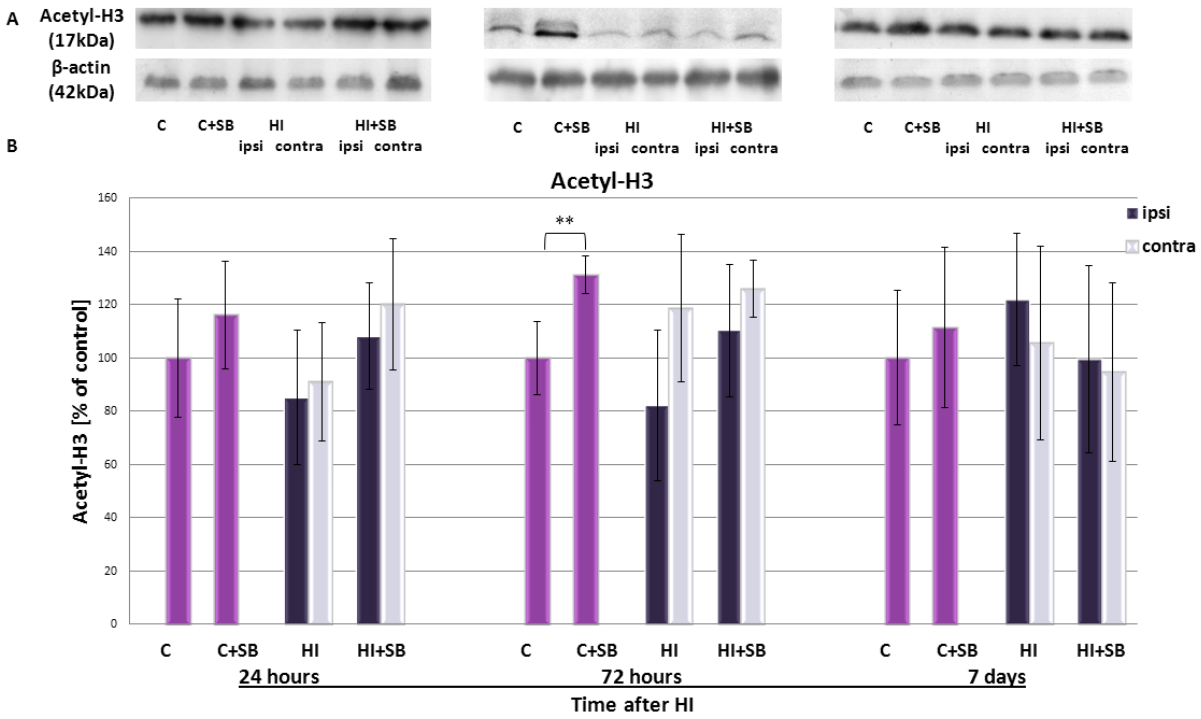


Fig.9. Effect of sodium butyrate on the expression of acetyl-H3 in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of Acetyl-H3. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, **p<0.01. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.3. Time course of cell proliferation in the rat brain

The time course of cell proliferation in two neurogenic areas – the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) was studied at specific time points after hypoxic-

ischemic injury (3, 6, 9 and 11 days). For this purpose, animals received a single dose of BrdU 24 hours prior to sacrifice. The number of newly generated cells was determined in the entire DG region of the hippocampus and in the SVZ by monitoring the incorporation and subsequent immunohistochemical detection of BrdU.

4.3.1. Cell proliferation in the dentate gyrus of the hippocampus

As evidenced by the analysis of hippocampal sections, there was no difference in the number of proliferating cells (BrdU+) between injured and control, sham-operated animals. The highest density of BrdU-labelled cells was detected between 3 and 6 days of recovery. Thereafter, cell proliferation decreased markedly in all experimental groups, almost 7-fold, as compared with the primary time point, indicating lowering of the dynamic of stem/progenitor cell proliferation (Fig. 10A).

The distribution of BrdU labeled cells throughout the brain varies between the topographical areas. At 3 days of recovery a great number of dividing cells was seen in the hilus area. With the prolongation of recovery to 9 and 11 days BrdU+ cells are located almost exclusively in the neurogenic subgranular zone (SGZ) of the DG (Fig. 10B). Interestingly, the exposure to SB did not affect the number of BrdU-positive cells in DG - neither in ipsi- nor in contralateral side (not shown).

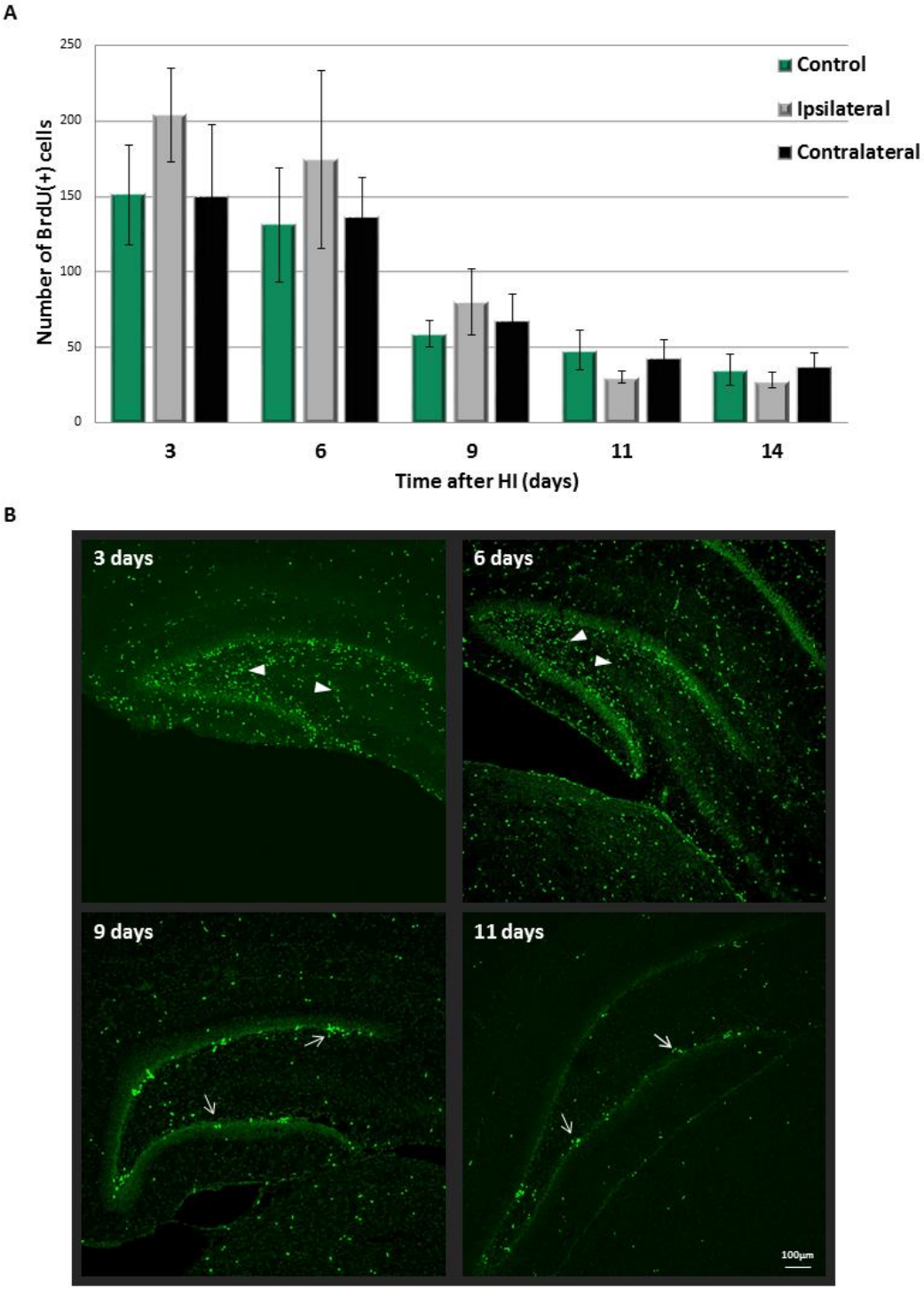


Fig.10. Time course of cell proliferation in the dentate gyrus of the hippocampus. (A) The number of BrdU-labeled nuclei within the DG area in sham-operated control animals and at different times after hypoxia-ischemia. The values are means ± SD from 5 animals per group and time point. One-way ANOVA and Bonferroni test did not indicate significant differences between experimental groups. (B) Newly divided cells in the HI-injured ipsilateral DG of the rat hippocampus. Confocal photomicrographs show immunohistochemical reactions at 3, 6, 9, and 11 days after HI. While at 3 days of recovery the greatest number of BrdU-positive cells is seen in the hilus (arrowheads), with the prolongation of recovery time, their number becomes particularly pronounced in the neurogenic SGZ (arrows) of the hippocampus.

4.3.2. Cell proliferation in the subventricular zone

The highest number of BrdU-positive cells within the SVZ area was noted between 3 and 6 days of recovery, similarly to the DG. Confocal analysis of brain sections revealed BrdU immunoreactive cells concentrated in the SVZ; however a number of these cells were also distributed throughout the adjacent corpus callosum and striatum. As depicted in Fig. 11, the intensity of BrdU labelling in the analyzed experimental groups was maintained at the same level. The significant decrease in the number of dividing cells in the ipsi-, as well as in the contralateral SVZ of HI animals compared to control ($p<0.001$), was only seen 9 days after the injury. The prolongation of recovery to 11 days led to reduction in proliferated cell number by about 40% in average in relation to the first investigated time point. Likewise, the exposure to SB did not affect the number of BrdU cells (not shown).

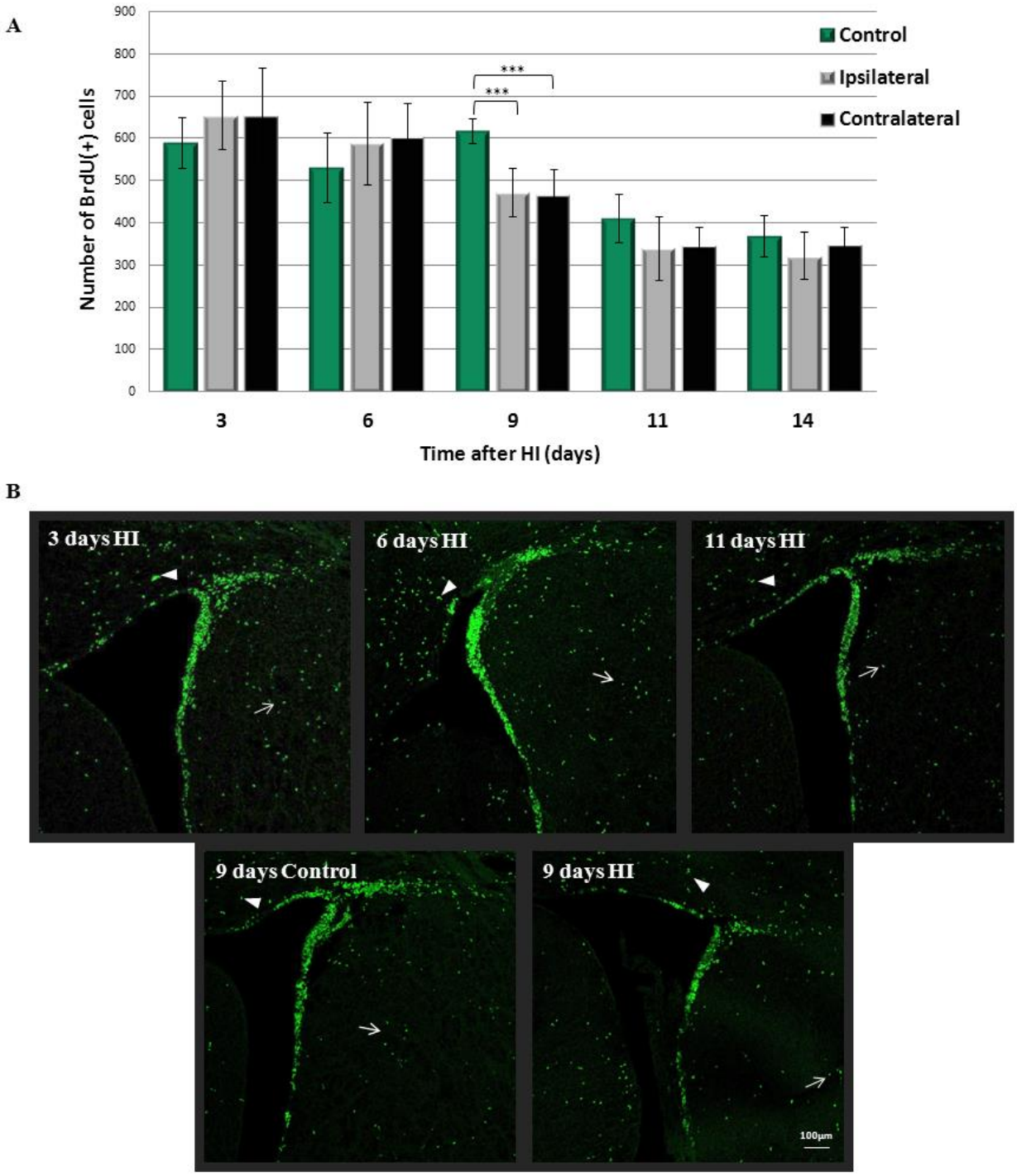


Fig.11. Time course of cell proliferation in the subventricular zone. (A) The number of BrdU-labeled nuclei within the SVZ area in sham-operated control animals and at different time points after hypoxia-ischemia. The values are means ± SD from 5 animals per group and time point. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, ***p<0.001. (B) Newly divided cells in the HI-injured ipsilateral and control SVZ, as well as in the corpus callosum (arrowheads) and striatum (arrows). Confocal photomicrographs show immunohistochemical reactions at 3, 6, 9, and 11 days after HI.

4.4. Phenotypic characterization of proliferating cells after neonatal hypoxia-ischemia

To further characterize the fate of newly arisen cells after SB treatment, brain tissue sections from sham and HI rats were double-stained for BrdU and different neural antigens – doublecortin (DCX; for neuroblasts), calbindin (for mature granule neurons), NeuN (for mature neurons), NG2 (detected in oligodendrocyte progenitor cells), O4 (for immature, non-myelinating oligodendrocytes), ED1 (for microglia) and GFAP (for astrocytes). For the purpose of these studies, animals received multiple BrdU injections on days 4-6 after hypoxia-ischemia and were sacrificed at 14 or 28 days of recovery

4.4.1. Phenotypic characterization of proliferating cells in the dentate gyrus of the hippocampus

Immunofluorescence assays using BrdU-labeling revealed no differences in the number of newly generated cells between experimental groups two weeks after HI, regardless of SB treatment. Double fluorescent studies showed numerous BrdU positive nuclei in the neurogenic subgranular zone of DG closely associated with neuronally committed precursors and/or immature neurons (neuroblasts) expressing a microtubule associated protein – doublecortin (DCX) (Fig. 12A). DCX-positive cells were extensively distributed in the DG of sham-operated control animals, as well as of the group treated with SB. As shown by labeling and subsequent counts, the number of BrdU/DCX+ cells was significantly decreased (by about 50%) in the hypoxic-ischemic side at 14 days of recovery compared with controls (mean counts 24 vs. 50 BrdU/DCX positive cells, respectively; $p<0.05$). In contrast, the number of cells in the side contralateral to ligation, although exposed to hypoxia, remained close to the controls (Fig. 12B). It clearly appears that the administration of sodium butyrate substantially increased BrdU/DCX positive cells in HI side to the value presented in sham. In the same conditions the number of neuroblasts slightly increased also within the DG of the hypoxic side; however the changes were not significant as compared to the vehicle–treated animals.

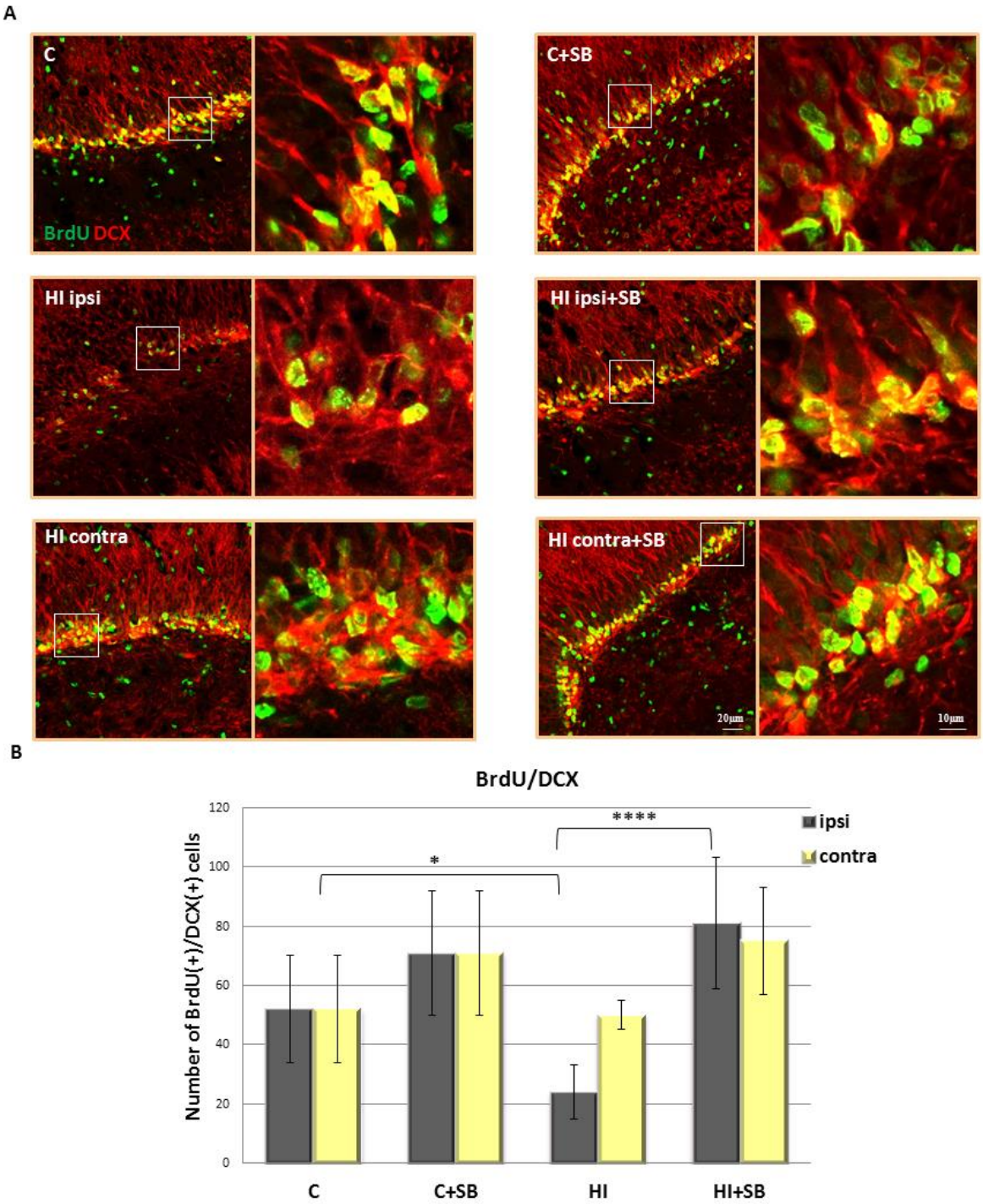


Fig.12. Effect of sodium butyrate on the generation of neuroblasts in the dentate gyrus of the hippocampus. (A) Brain sections from control animals and from animals 14 days after HI were stained for BrdU immunoreactivity (green) and for a neuroblast marker-DCX (red). Confocal photomicrographs show double-labelled cells (yellow) in the DG with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/DCX labelled cells. (B) Number of BrdU/DCX-positive cells quantified in the DG area (0.36 mm^2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05 and ****p<0.0001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

To check whether neuroblasts localized in the subgranular zone of DG differentiate to mature neurons, double labeling assay using BrdU with calbindin – a marker of granule neuronal cells was performed (Fig. 13A). Quantified results are shown on Fig. 13B. It clearly appears that 28 days after hypoxic-ischemic injury there is a significant decrease (more than 50%) in double stained BrdU/Calbindin+ mature granule neurons in the DG region of the ipsilateral (hypoxic-ischemic) side compared with controls ($p<0.001$), without significant effect noticed contralaterally. In contrast to the beneficial effect of SB on the number of neuroblasts in the ipsilateral DG, in the same applied conditions, the inhibitor did not increase the amount of new-generated granule neurons, which remained persistently lower than control.

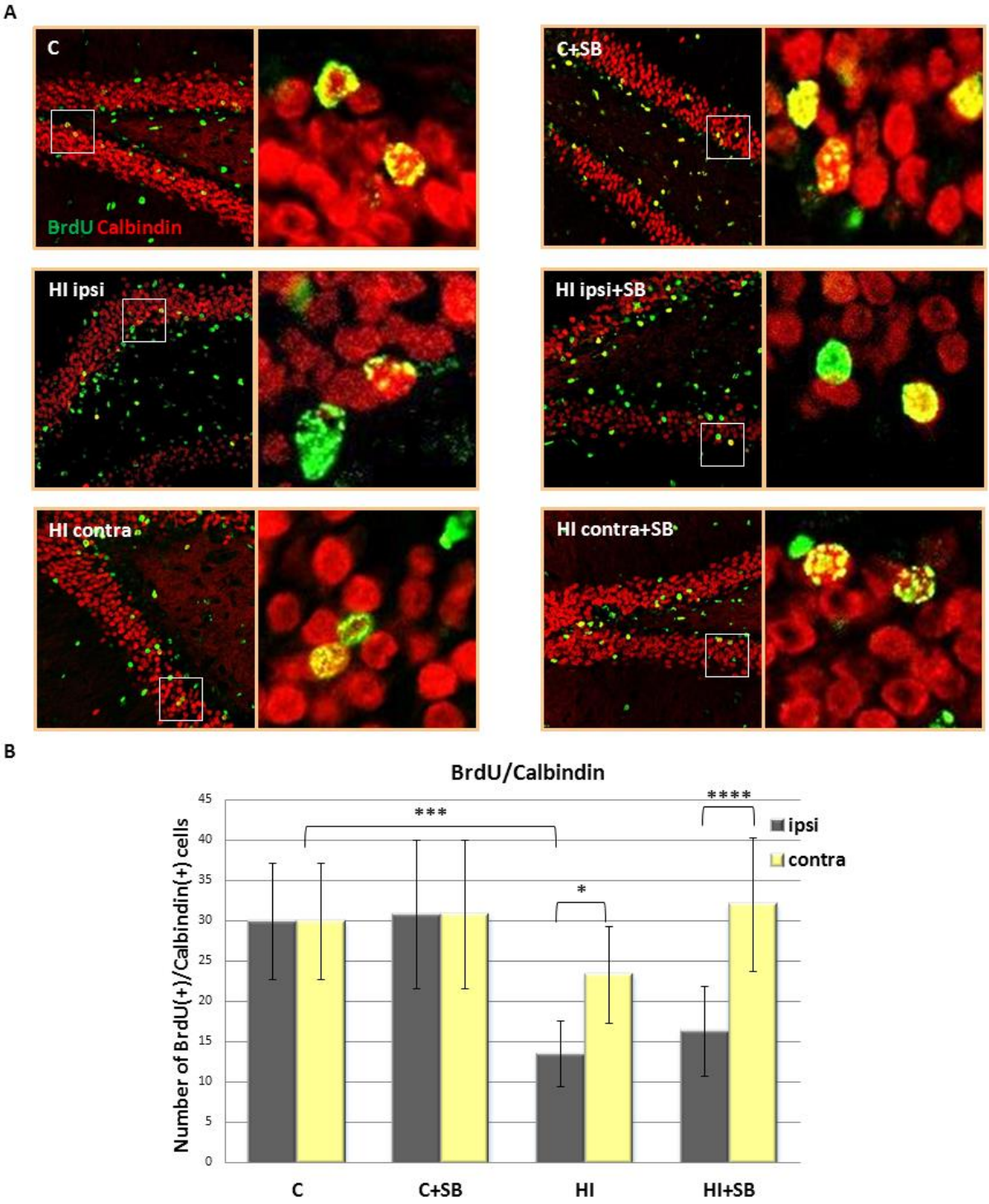


Fig.13. Effect of sodium butyrate on the generation of mature neurons in the dentate gyrus of the hippocampus. (A) Brain sections from control animals and from animals 28 days after HI were stained for BrdU immunoreactivity (green) and for granule neuron marker–Calbindin (red). Confocal photomicrographs show double-labelled cells (yellow) in the DG with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/Calbindin labelled cells. (B) Number of BrdU/Calbindin-positive cells quantified in the DG area (0.36 mm2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05, ***p<0.001 and ****p<0.0001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

In order to evaluate differentiation of newly produced cells to oligodendroglial phenotype, double fluorescent studies for BrdU and specific markers was carried out. To address the question whether SB stimulates oligodendrogenesis within the DG area, the number of proliferating oligodendrocyte precursor cells (OPCs) (Fig. 14A) as well as more mature oligodendrocytes (Fig. 15A) in sham and hypoxic-ischemic animals after 14 and 28 days was analyzed. For this purpose, antibodies specific for cells being at a different developmental stage were used. In control a number of BrdU-labeled cells co-stained with NG2, commonly used for the identification of OPCs was observed. As is shown in Fig. 14B, 14 days after the hypoxic-ischemic insult, a marked decrease in new OPCs (by about 75 % compared to sham control) ipsilaterally ($p<0.0001$) was noted; while the number of these cells in the contralateral hypoxic side did not present statistical significance from the control. Exposure to SB resulted in a significant raise in the number of new progenitors in the ipsilateral ($p<0.001$) as well as in the contralateral ($p<0.01$) side. The restoration of the lost OPCs to the level of sham control and maintained further at 28 days of recovery could be due, at least in part, to the neuroprotective effect of this agent. At the same time point (28 days), the density of cells double-stained with BrdU/O4 (marker of the later-stage progenitor cells) in injured animals did not differ from control group. In addition there was no visible effect of SB on the number of these cells (Fig. 15B).

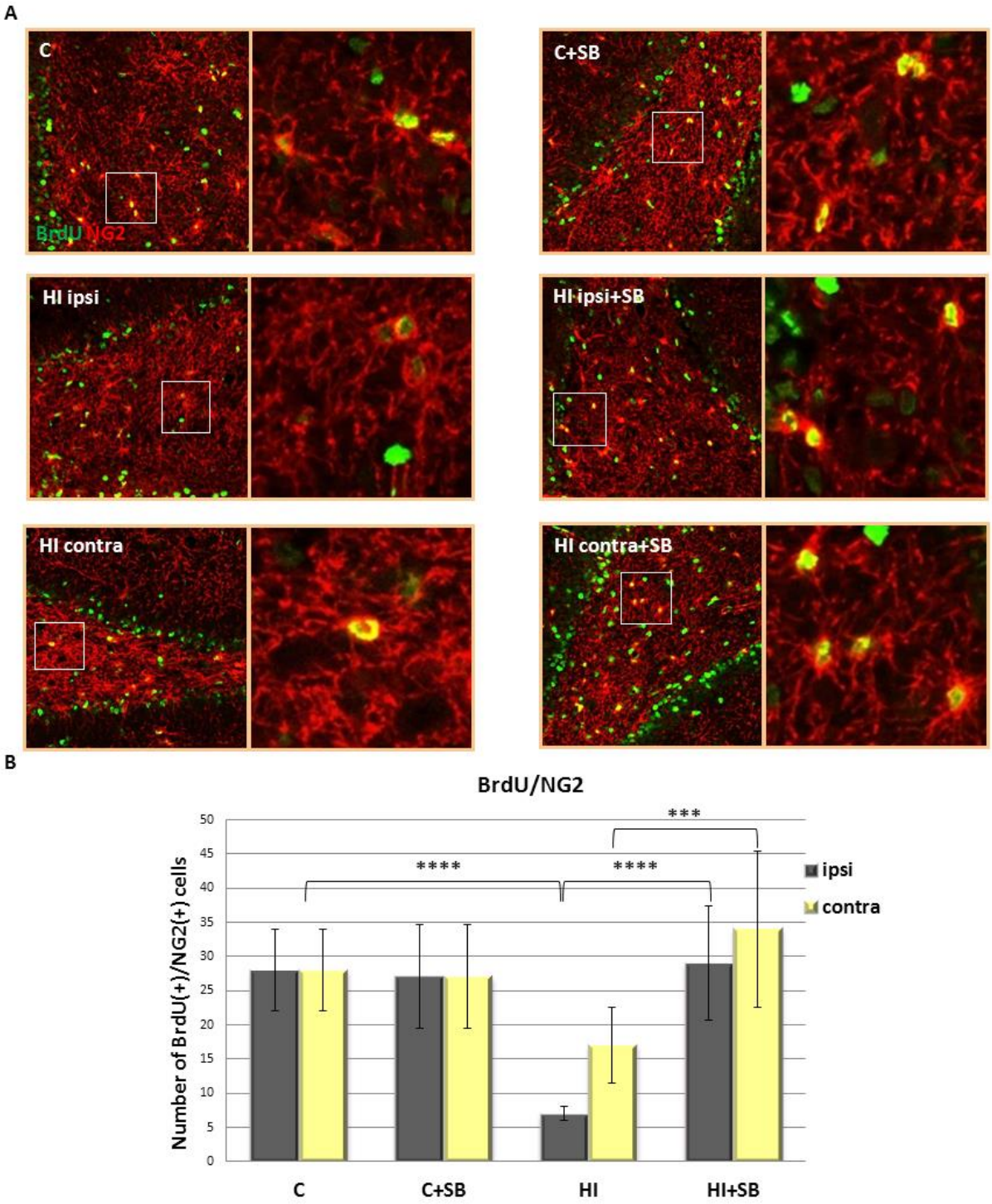


Fig.14. Effect of sodium butyrate on oligodendrocyte precursor cell proliferation in the dentate gyrus of the hippocampus. (A) Brain sections from control animals and from animals 14 days after HI were stained for BrdU immunoreactivity (green) and for OPC marker – NG2 (red). Confocal photomicrographs show double-labelled cells (yellow) in the DG with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/NG2 labelled cells. (B) Number of BrdU/NG2-positive cells quantified in the DG area (0.36 mm2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, ***p<0.001 and ****p<0.0001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

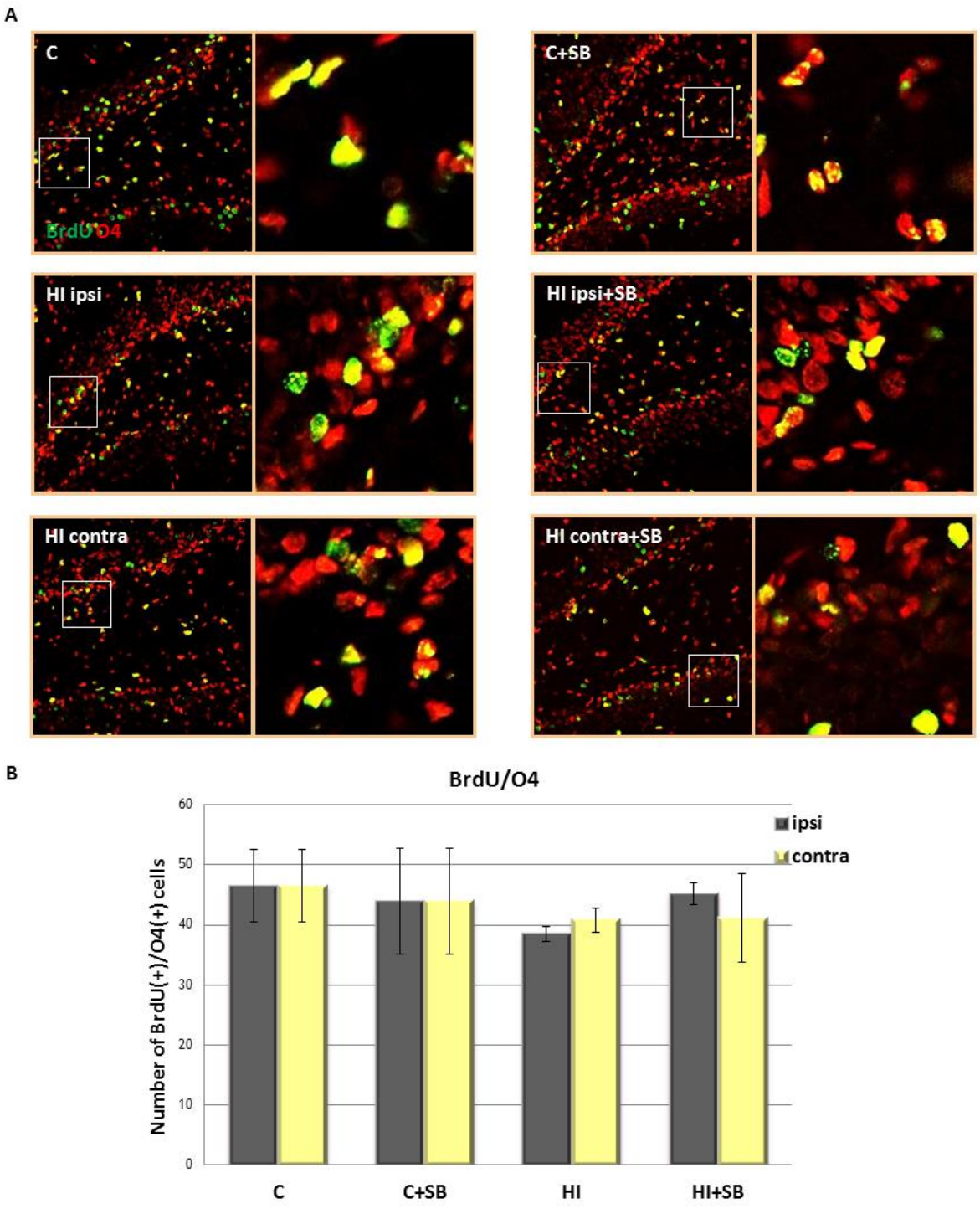


Fig.15. Effect of sodium butyrate on the generation of non-myelinating oligodendrocytes in the dentate gyrus of the hippocampus. (A) Brain sections from control animals and from animals 28 days after HI were stained for BrdU immunoreactivity (green) and for oligodendrocyte marker–O4 (red). Confocal photomicrographs show double-labelled cells (yellow) in the DG with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/O4 labelled cells. (B) Number of BrdU/O4-positive cells quantified in the DG area (0.36 mm2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test did not indicate significant differences between experimental groups. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.4.2. Phenotypic characterization of proliferating cells in the subventricular zone

Hypoxic-ischemic injury, without as well as with SB treatment exerted no effect on the number of dividing cells (BrdU positive) 14 days after the insult, compared with the sham group. To determine if proliferating cells progressed to form neural progenitors, BrdU labelled cells were stained with DCX, a marker for neuroblasts.

Representative photomicrographs of the immunohistochemistry using brains 14 days after HI and respective quantitative data are shown in Fig. 16. Surprisingly, and conversely to the SGZ findings, HI injury did not change the quantity of BrdU/DCX-positive cells distributed extensively within the SVZ area. The only notable change found in the SVZ was a marked increase in the number of neuroblasts in the ipsilateral, hypoxic-ischemic hemisphere after SB administration compared to control or HI untreated with SB ($p<0.001$).

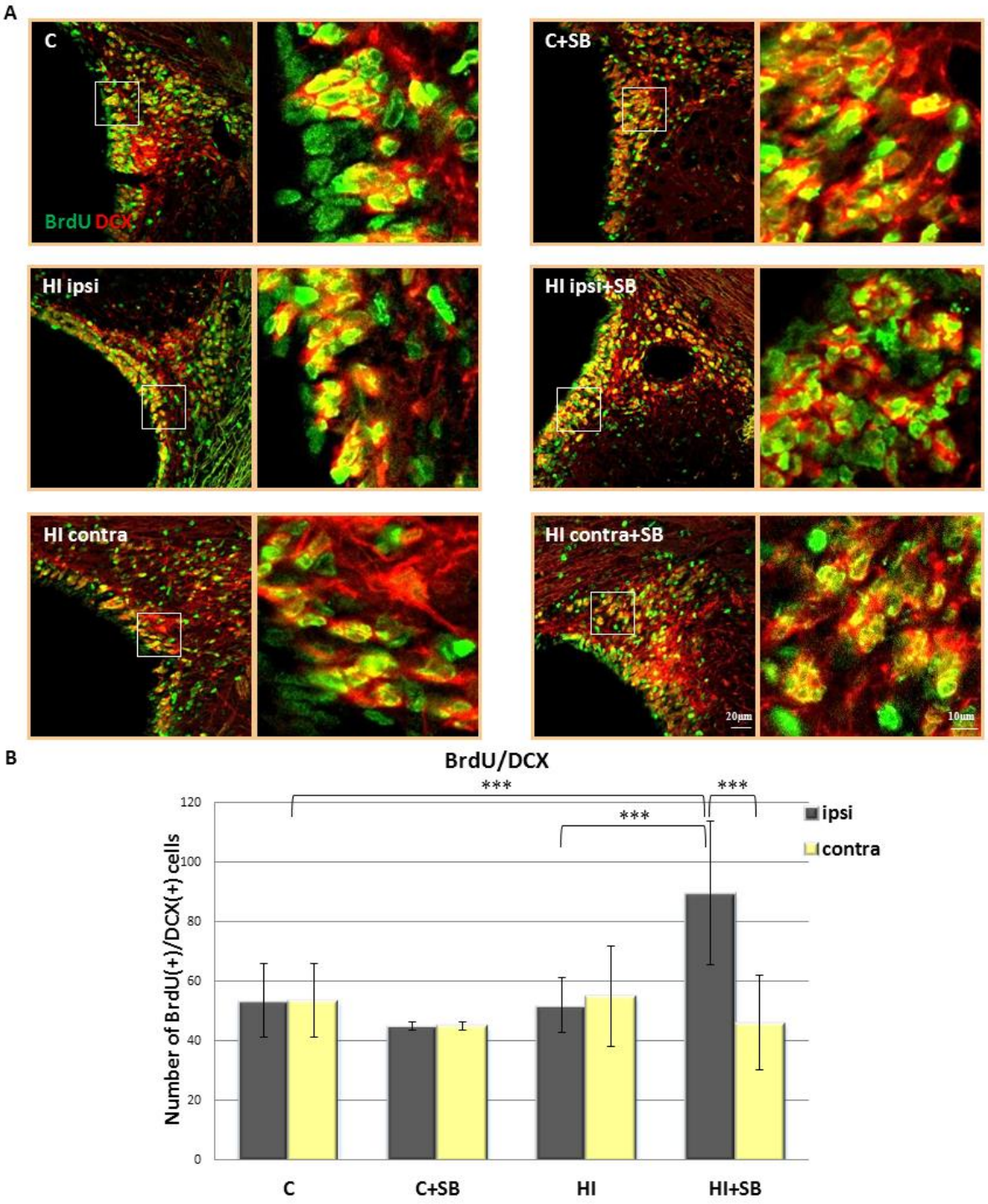


Fig.16. Effect of sodium butyrate on the generation of neuroblasts in the subventricular zone. (A) Brain sections from control animals and from animals 14 days after HI were stained for BrdU immunoreactivity (green) and for a neuroblast marker-DCX (red). Confocal photomicrographs show double-labelled cells (yellow) in the SVZ with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/DCX labelled cells. (B) Number of BrdU/DCX-positive cells quantified in the SVZ area (0.36 mm^2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

To investigate whether neuroblasts identified in the SVZ differentiate into mature neurons, brain sections were examined for co-localization of BrdU with mature neuronal cell-type specific marker – NeuN (Fig. 17A). The number of double labeled cells in the left SVZ of lesioned animals at 28 days of recovery did not differ significantly from the control group (Fig. 17B). However a tendency for the number of double labeled cells to decrease could be observed. The administration of SB after HI significantly increased the density of newly formed neurons in the SVZ of the injured hemisphere as compared to the untreated animals ($p<0.001$), yet these changes did not differ from the control group. This finding provided evidence that SB treatment increased the generation of new neurons in the injured HI hemisphere. A subset of BrdU/NeuN positive cells was also observed in the striatum, however their number estimated in all experimental groups was maintained on similar level.

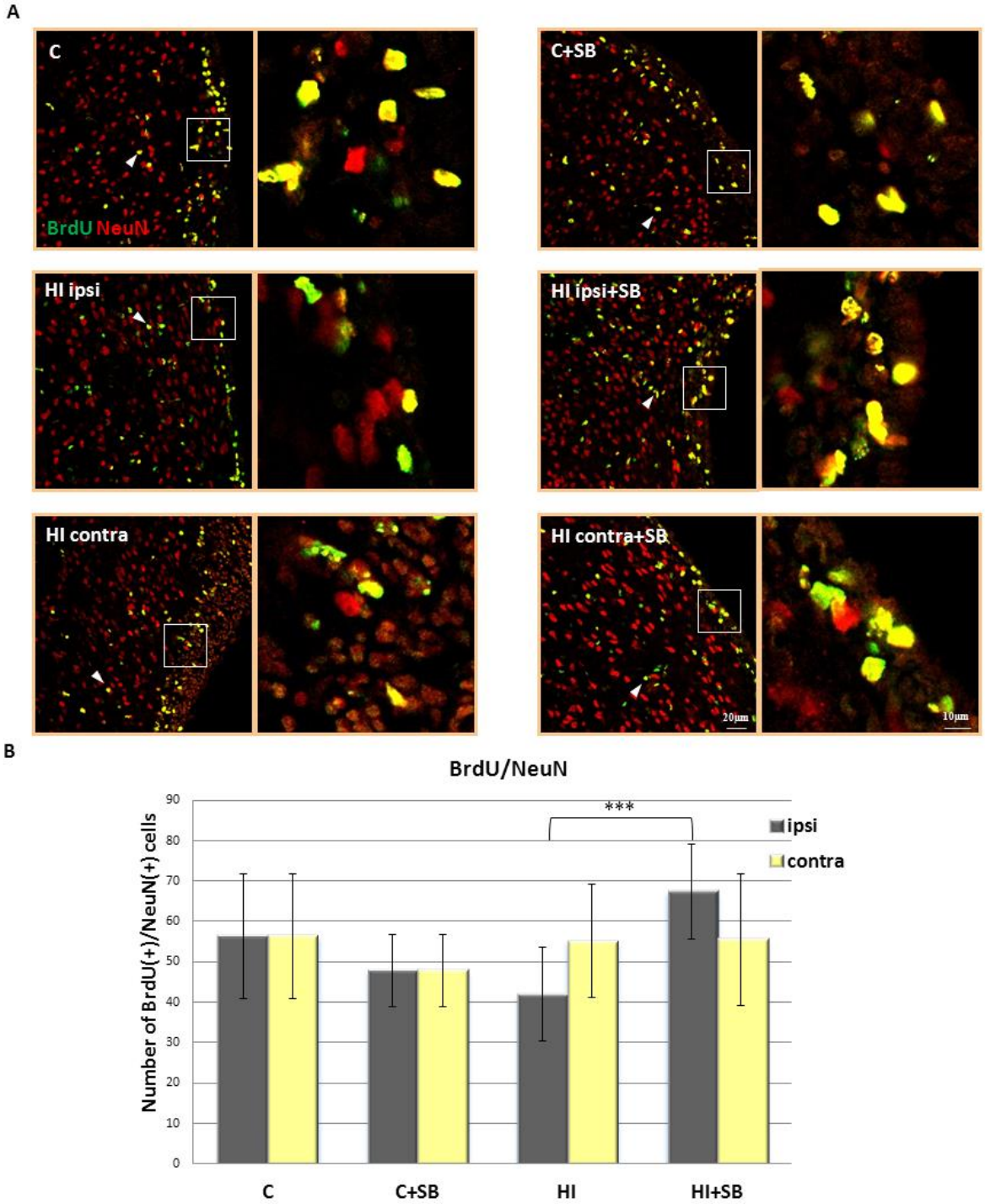


Fig.17. Effect of sodium butyrate on the generation of neurons in the subventricular zone. (A) Brain sections from control animals and from animals 28 days after HI were stained for BrdU immunoreactivity (green) and for a neuron marker–NeuN (red). Confocal photomicrographs show double-labelled cells (yellow) in the SVZ with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/NeuN labelled cells. Arrowheads indicate BrdU/NeuN+ cells in striatum. (B) Number of BrdU/NeuN-positive cells quantified in the SVZ area (0.36 mm^2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

In order to explore responses of the oligodendrocyte progenitor population, double fluorescence studies were carried out with BrdU and glial antibodies specific for particular stages of oligodendrocyte development – anti-NG2 for identification of oligodendrocyte progenitors, and anti-O4 for immature, however more developed, non-myelinating oligodendrocytes. Sections immunostained with anti-NG2 showed few double labelled cells in the SVZ area (Fig. 18A) 14 days after HI. There were no significant differences in the number of BrdU/NG2-positive cells between investigated groups, however a clear decreasing tendency, pronounced particularly in the ipsilateral side, was observed (Fig. 18B). Moreover, neither HI alone nor HI together with SB treatment influenced the number of non-myelinating oligodendrocytes (BrdU/O4-positive). However, detailed confocal analysis of multiple sections indicated a very subtle tendency for the number of these cells to reduce in both hemispheres after HI (Fig. 19). Many BrdU-labeled cells expressing NG2 or O4, indicating oligodendroglial lineage, were present on a stable level in the striatum.

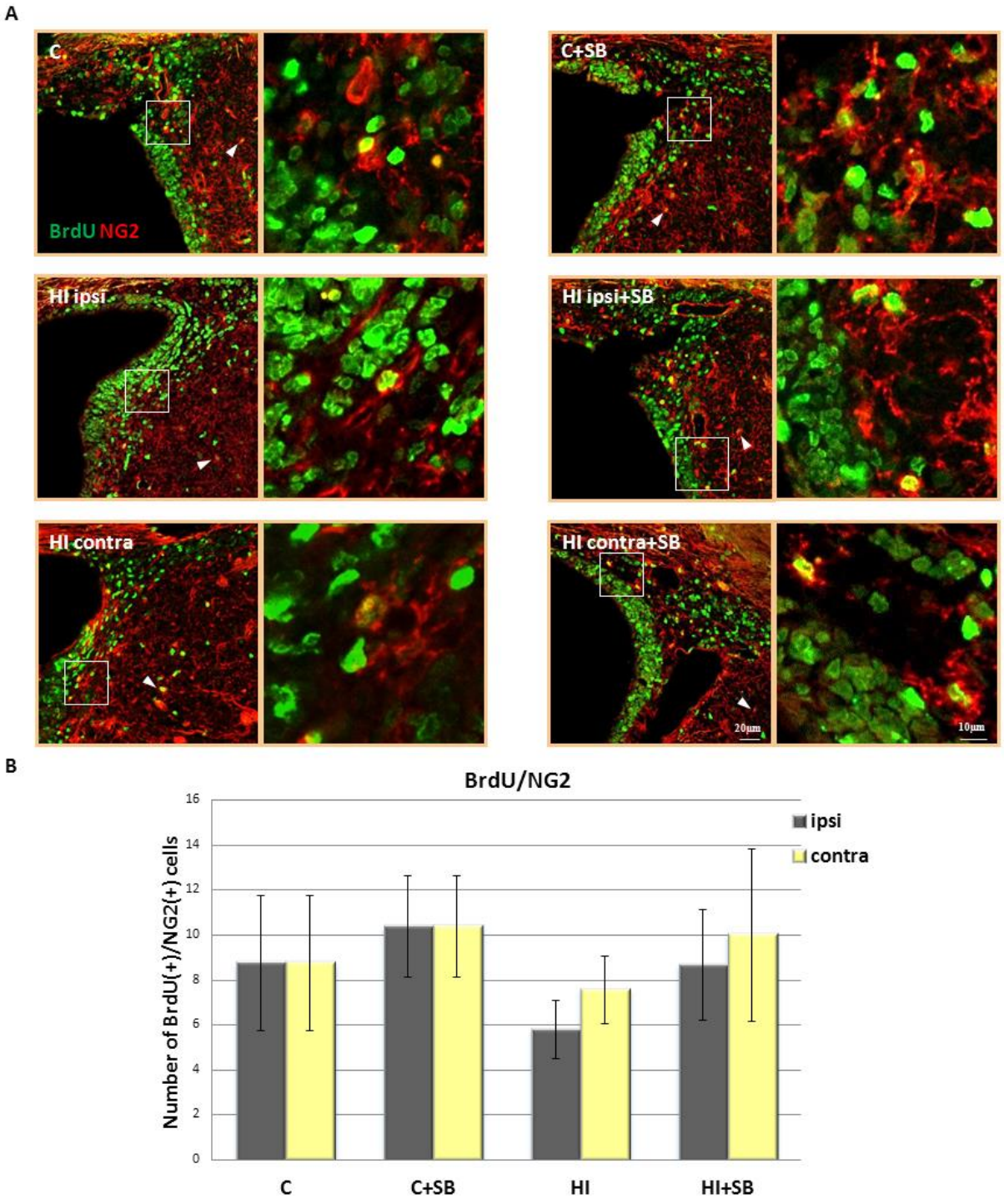


Fig.18. Effect of sodium butyrate on oligodendrocyte precursor cell proliferation in the subventricular zone. (A) Brain sections from control animals and from animals 14 days after HI were stained for BrdU immunoreactivity (green) and for OPC marker–NG2 (red). Confocal photomicrographs show double-labelled cells (yellow) in the SVZ with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/NG2 labelled cells. Arrowheads indicate BrdU/NG2+ cells in striatum. (B) Number of BrdU/NG2-positive cells quantified in the SVZ area (0.36 mm^2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test did not indicate significant differences between experimental groups. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

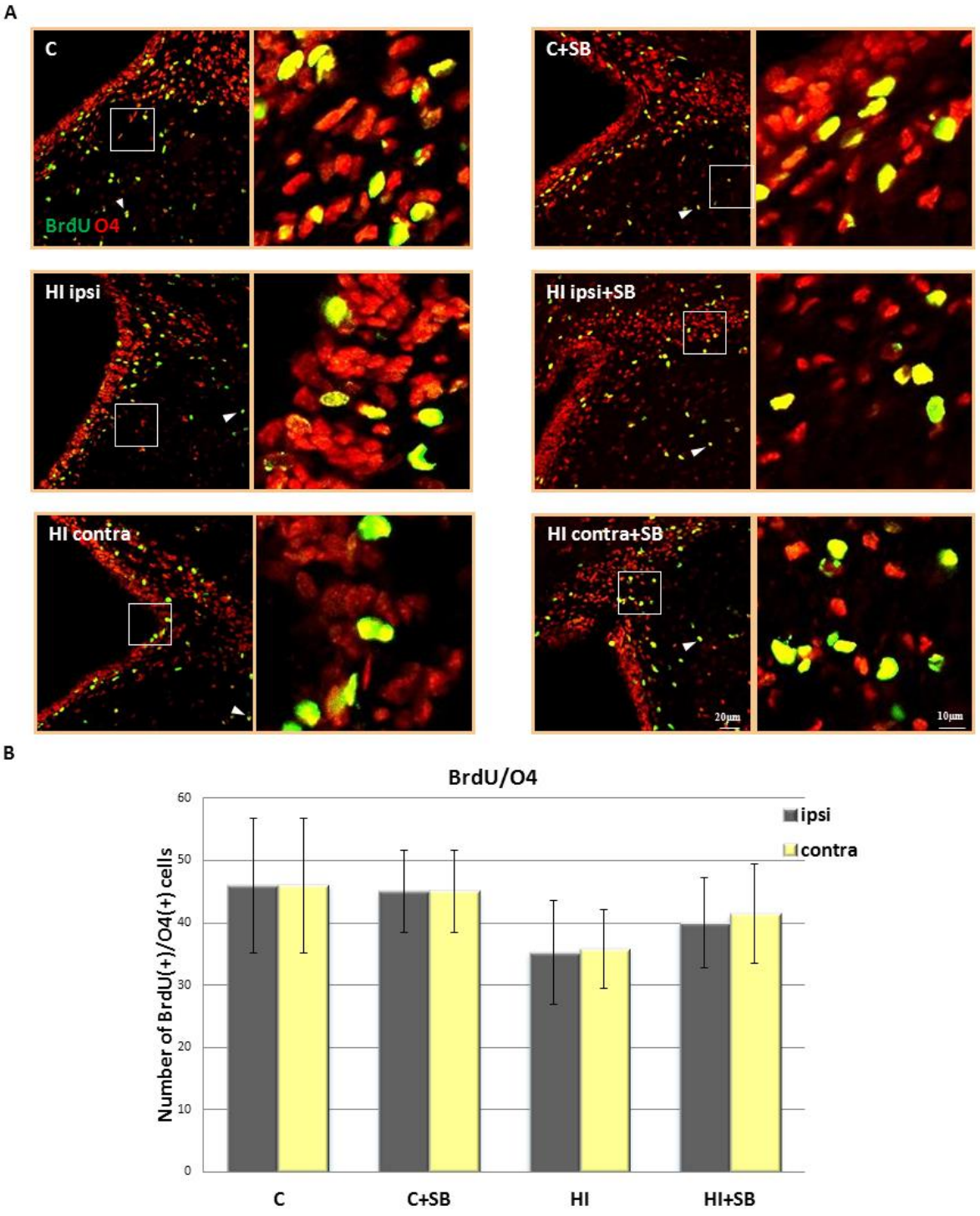


Fig.19. Effect of sodium butyrate on the generation of non-myelinating oligodendrocytes in the subventricular zone. (A) Brain sections from control animals and from animals 28 days after HI were stained for BrdU immunoreactivity (green) and for oligodendrocyte marker–O4 (red). Confocal photomicrographs show double-labelled cells (yellow) in the SVZ with or without SB treatment. Enlargements present areas marked in rectangles with respective BrdU/DCX labelled cells. Arrowheads indicate BrdU/O4+ cells in striatum. (B) Number of BrdU/O4-positive cells quantified in the SVZ area (0.36 mm2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test did not indicate significant differences between experimental groups. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.5. Effect of sodium butyrate on microglial/macrophage and astroglial response to neonatal hypoxia-ischemia

4.5.1. Microglial/macrophage response to neonatal hypoxia-ischemia

To determine the effect of SB administration on cerebral activation/influx of microglia/macrophages after hypoxia-ischemia, ED1 staining on brain sections of sham-operated, HI, and HI treated with SB rat pups was performed. The data presented in Fig. 20A shows numerous ED1-positive cells in ipsilateral hemisphere 14 days after HI. Most microglial/macrophage cells were round-shaped with thick processes and were considered to be in an activated state. The activated microglial/macrophage cells were scattered throughout the entire cortex and striatum. Contrary, in slices obtained from control animals, as well as from contralateral hemispheres, the activated microglial/macrophage cells were not detected. Sodium butyrate administration resulted in a decreased number of microglial cells to 15% of vehicle-treated animals in the ipsilateral side ($p<0.05$) (Fig. 20B).

Moreover, as depicted in Fig. 21 the decrease in the number of ED1 positive cells after SB administration to HI injured animals correlated with a reduction in newly generated microglia/macrophages, estimated by quantification of double labeled BrdU/ED1 cells ($p<0.001$).

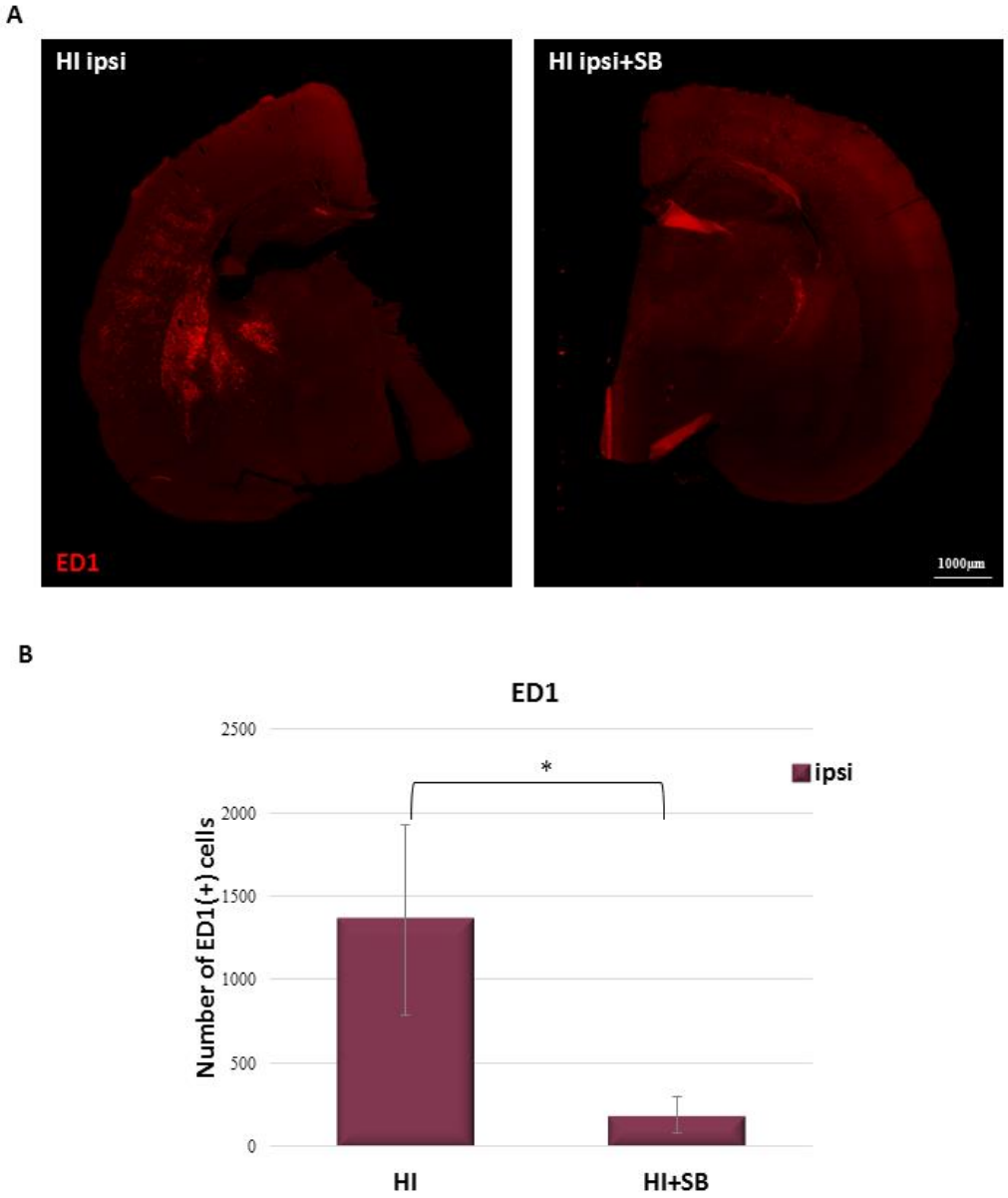


Fig.20. Effect of sodium butyrate on microglial/macrophage cell number in the ipsilateral hemisphere after neonatal hypoxia-ischemia. (A) Confocal photomicrographs of brain sections from animals 14 days after HI, with or without SB treatment, stained for activated microglia/macrophage marker – ED1 (red). (B) Number of ED1-positive cells quantified in whole hemisphere. The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05. Abbreviations: ipsi *ipsilateral*.

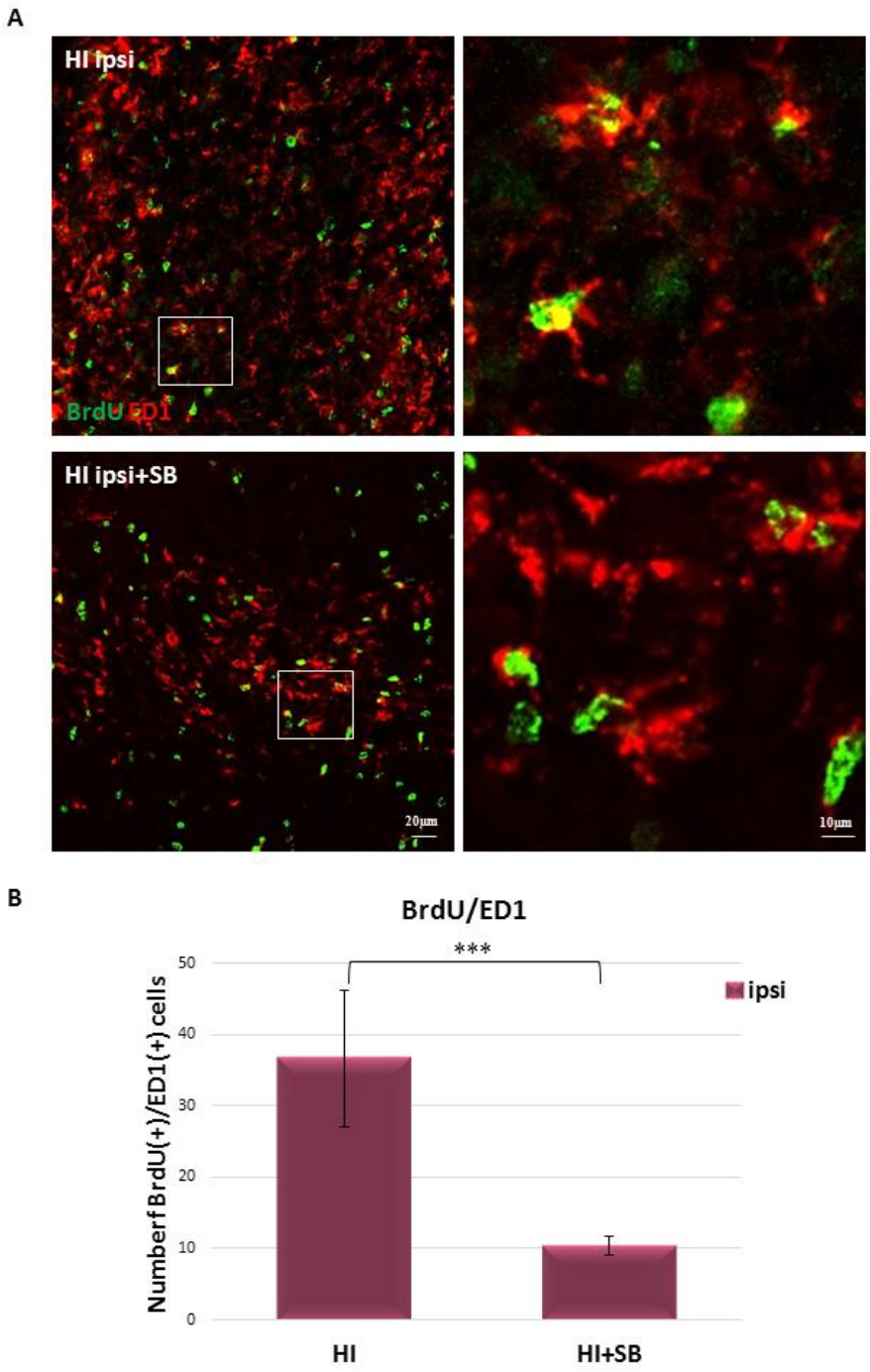


Fig.21. Effect of sodium butyrate on the generation of microglial/macrophage cells in the ipsilateral hemisphere after neonatal hypoxia-ischemia. (A) Confocal photomicrographs of brain sections from animals 14 days after HI, with or without SB treatment, stained for BrdU immunoreactivity (green) and for activated microglia/macrophage marker – ED1 (red). Enlargements present areas marked in rectangles with respective double labelled cells (yellow). (B) Number of BrdU/ED1-positive cells quantified in the ipsilateral hemisphere area (0.36 mm^2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, ***p<0.001. Abbreviations: ipsi *ipsilateral*.

In the next step the effect of SB on the polarization of microglia from M1 to M2-like phenotype after HI was assessed. To address this, double staining with IL-1β antibody coupled with ED1 for the identification of activated pro-inflammatory M1 phenotype (Fig. 22A) and ED1/arginase-1 for anti-inflammatory M2-like phenotype (Fig. 22B) was performed. Two weeks after HI, the majority of ED1 positive cells expressed IL-1β in the cortical region of the ipsilateral hemisphere, with only a few cells stained positively with ED1/Arg-1. The administration of SB after HI led to a marked decrease in the amount of cells presenting the M1 phenotype of microglia ($p<0.001$ ipsilateral vs. ipsilateral with SB) (Fig. 22C) with concomitant enhancement of cells stained with ED1/Arg-1 specific for M2 type ($p<0.01$ ipsilateral vs. ipsilateral with SB) (Fig. 22D).

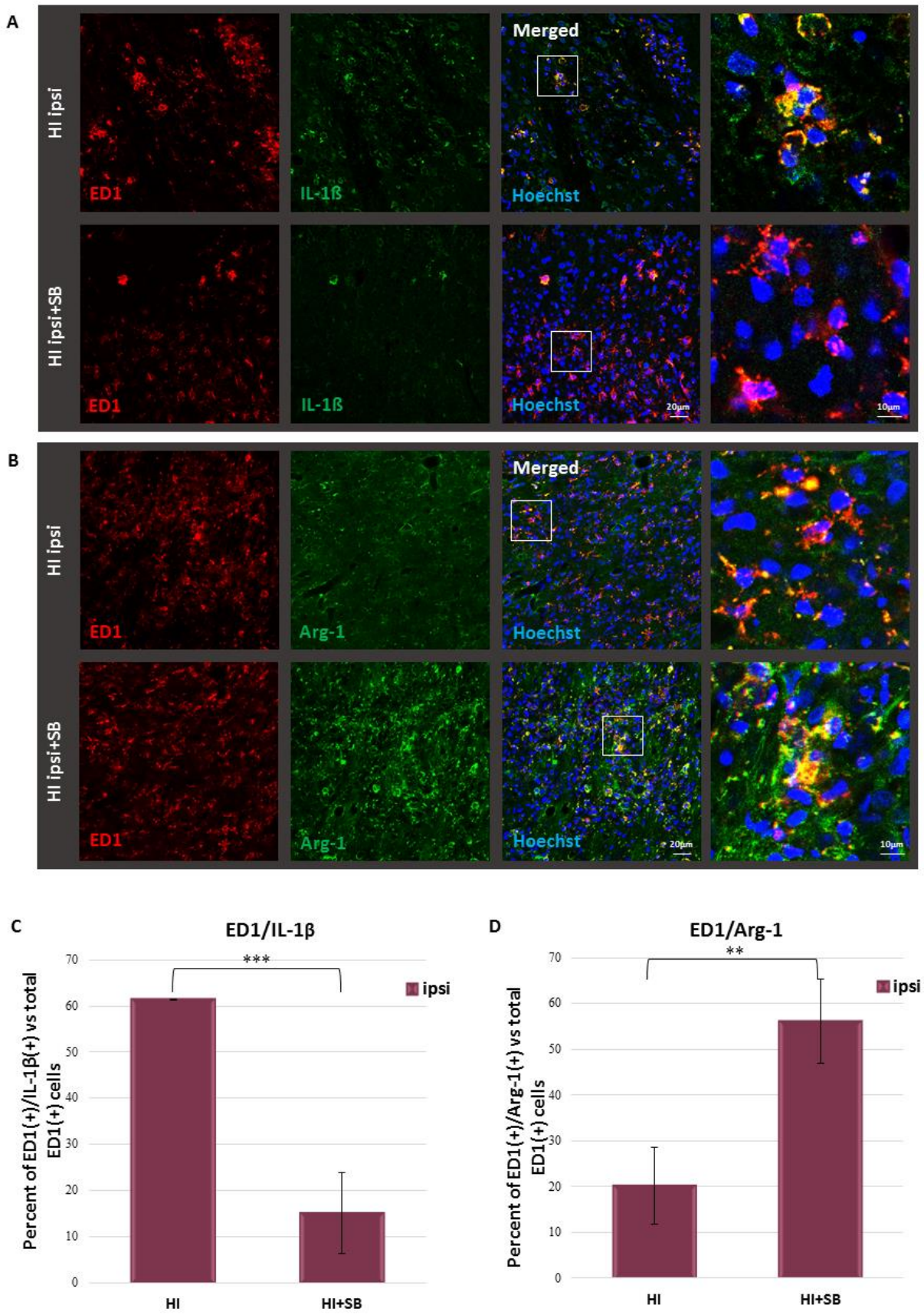


Fig.22. Effect of sodium butyrate on microglia/microphage phenotype in the ipsilateral hemisphere. Confocal photomicrographs of brain sections from animals 14 days after HI, with or without SB treatment, stained for

ED1 immunoreactivity (red), for IL-1β, marker for M1 phenotype (green) (A), and for arginase-1 (Arg-1), marker specific for M2 phenotype (green) (B). Nuclei were labeled with the Hoechst dye (blue). Enlargement of marked in rectangles areas show respective double labelled cells (yellow). Number of IL-1β/ED1-positive cells (C) and Arg-1/ED1-positive cells (D) quantified in the ipsilateral hemisphere area (0.36 mm^2). The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, **p<0.01 and ***p<0.001. Abbreviations: ipsi *ipsilateral*.

4.5.2. Astroglial response to neonatal hypoxia-ischemia

Subsequently, the density of astrocytic cells (GFAP+) was assessed. As depicted 14 days after neonatal HI, the GFAP-associated fluorescent signal increased in the ipsilateral hemisphere, compared to control group and to the contralateral side ($p<0.001$) (Fig. 23). As seen on microphotographs of brain slices, astrocytes were mainly observed in the cortex, striatum and SVZ. GFAP-positive cells presented an activated phenotype characterized by hypertrophic processes. SB treatment resulted in a significant increase in GFAP staining intensity by 36% in the ipsilateral hemisphere ($p<0.05$). The increase in staining was noted mainly in the striatum (Fig. 23A). In addition, the hypertrophy of astrocytic cells was more pronounced and associated with interdigitations of processes that overlapped and formed glial scars.

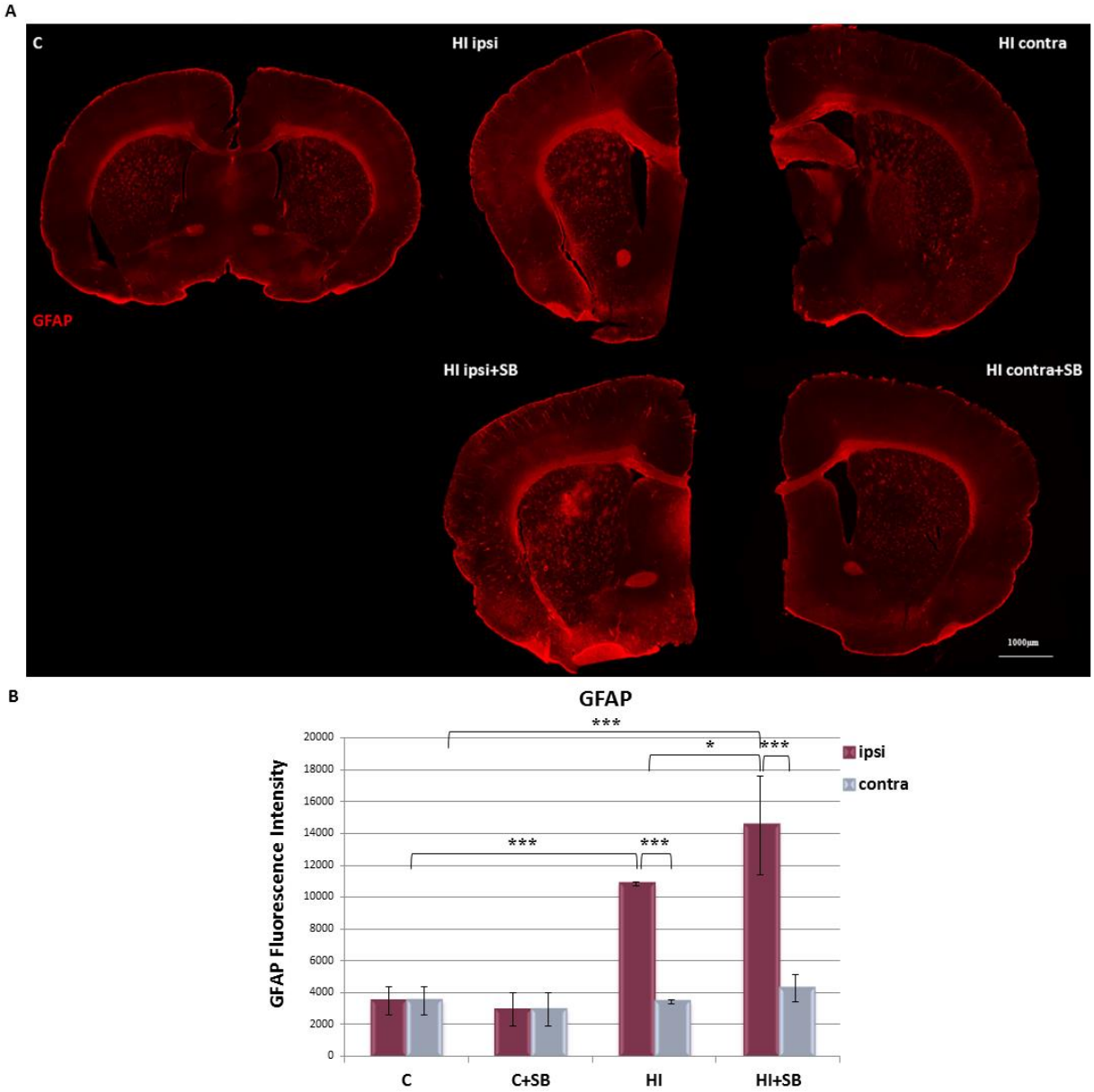


Fig.23. Effect of sodium butyrate on astrocytes in the brain. (A) Confocal photomicrographs of brain sections from animals 14 days after HI, with or without SB treatment, stained for astrocyte marker – Glial fibrillary acidic protein (GFAP; red). (B) GFAP fluorescence intensity quantified in whole hemisphere. The values are means ± SD from 5 animals per group. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.6. Contribution of neurotrophins to sodium butyrate-induced neurogenesis

The purpose of this part was to investigate whether the effect of SB on the generation of new cells, found in the current study, might be associated with the upregulation of neurotrophins (BDNF, pro- and mature form, and NGF).

4.6.1. *Effect of sodium butyrate on Brain-Derived Neurotrophic Factor (BDNF)*

As indicated in Fig.24 in each experimental condition the level of BDNF, estimated by ELISA, was significantly higher at postnatal day 14 (corresponding to 7 days of recovery) as compared to P10 (3 days of recovery) by about 36% on average. After hypoxic-ischemic injury the amount of BDNF remained close to the control value in both investigated time points. Animal treatment with SB led to an increase of BDNF level in the ipsilateral side 7 days after HI ($p<0.05$).

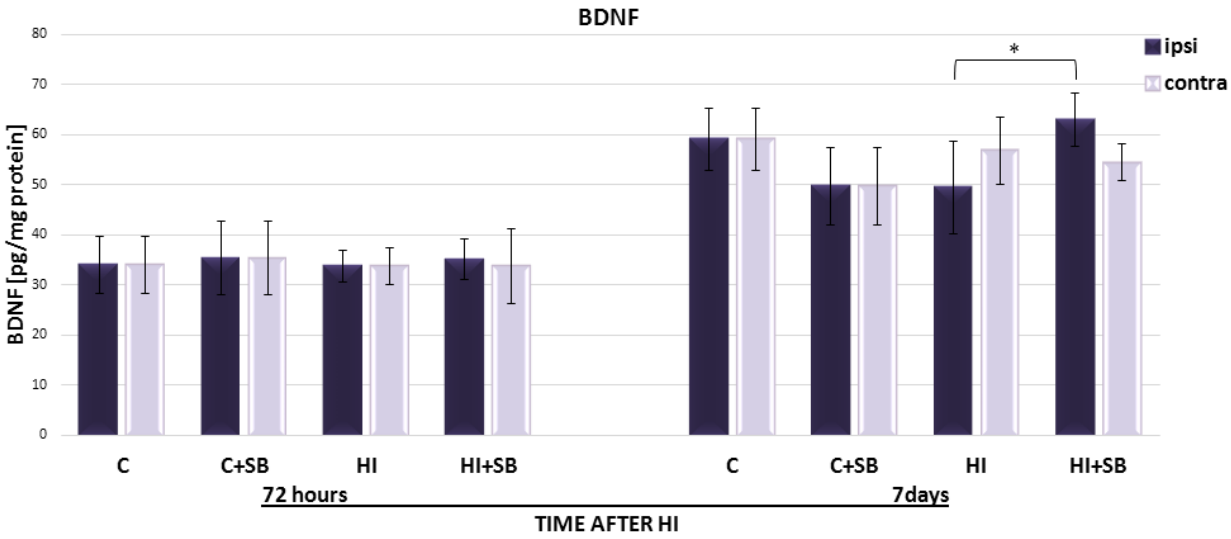


Fig.24. Effect of sodium butyrate on the expression of Brain-Derived Neurotrophic Factor in the brain after neonatal hypoxia-ischemia. Bar graph shows statistical analysis of BDNF protein level. The values are means ± SD from 5 animals per group and time point assessed in triplicates. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.6.2. Effect of sodium butyrate on pro-Brain-Derived Neurotrophic Factor (pro-BDNF)

A parallel set of samples was used for pro-BDNF assay, the precursor of mature BDNF. The results of immunoblot densitometry revealed that 24 hours and 7 days after HI there was a significant increase in the level of this pro-neurotrophin in the ipsilateral hemisphere after SB administration compared to the untreated injured side ($p<0.05$ after 24 hours and $p<0.001$ after 7

days). Unexpectedly, 72 hours post-injury the expression of pro-BDNF presented almost the same value in all investigated groups (Fig. 25).

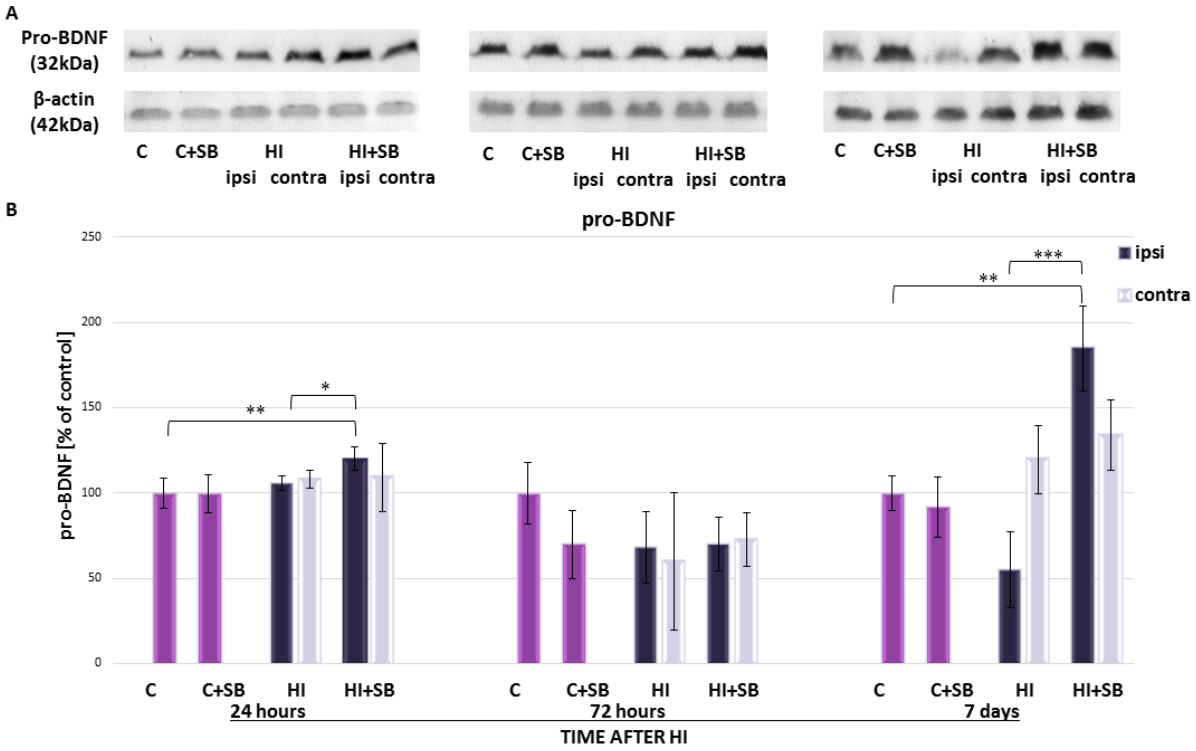


Fig.25. Effect of sodium butyrate on the expression of pro-Brain-Derived Neurotrophic Factor in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of pro-BDNF protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups,*p<0.05, **p<0.01 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.6.3. Effect of sodium butyrate on Nerve Growth Factor (NGF)

The expression of NGF was evaluated by ELISA. Similar to BDNF, in each experimental condition the level of NGF was significantly higher at postnatal day 14 (7 days of recovery) compared to P10 (3 days of recovery) (Fig. 26). A significantly elevated level of NGF was observed in the ipsilateral hemispheres (with as well as without SB treatment) 3 days after HI compared to control ($p<0.01$). With the prolongation of time to 7 days, the immunoreactivity of NGF markedly decreased in the hypoxic-ischemic side ($p<0.01$ ipsilateral vs. control), however it returned to control value after exposure to SB. In contrast, the level of NGF in contralateral sides remained close to the value presented by sham throughout the experiment.

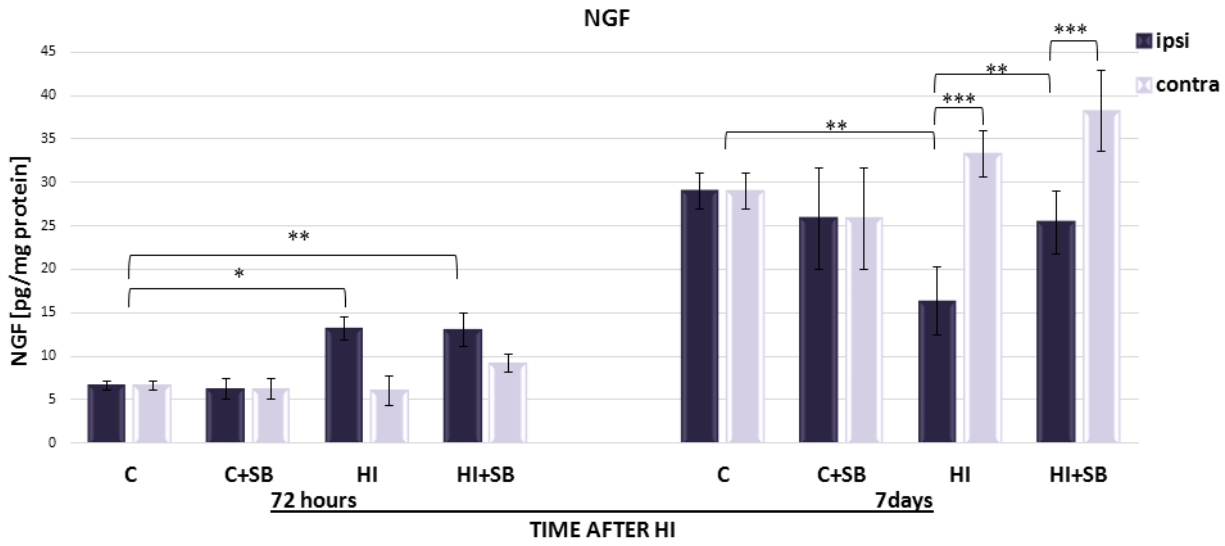
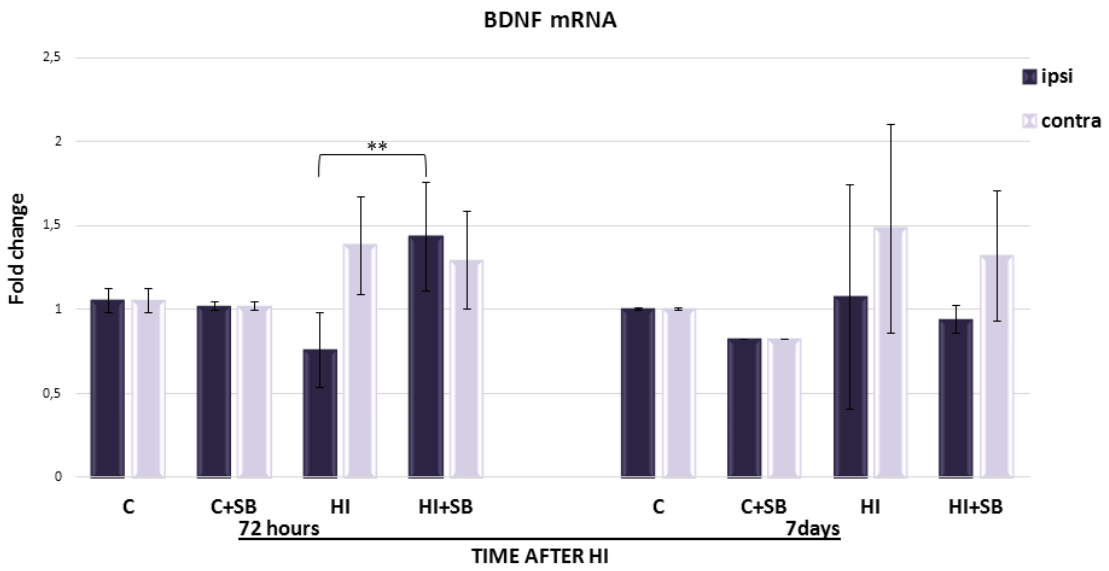


Fig.26. Effect of sodium butyrate on the expression of Nerve Growth Factor in the brain after neonatal hypoxia-ischemia. Bar graph shows statistical analysis of NGF protein level. The values are means ± SD from 5 animals per group and time point assessed in triplicates. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups,*p<0.05, **p<0.01 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.6.4. Effect of sodium butyrate on mRNA of Brain-Derived Neurotrophic Factor and Nerve Growth Factor

To investigate if the pattern of protein concentration changes of BDNF and NGF are similar to that presented by the expression of their messenger RNA (mRNA) qRT-PCR was performed. As shown in Fig. 27A SB treatment led to a remarkable increase in BDNF gene expression in the ipsilateral hemisphere 72 hours after HI ($p<0.01$ ipsilateral vs. ipsilateral with SB). No other considerable changes were noticed. Similarly, the expression of NGF gene did not show significant differences between investigated groups (Fig. 27B).

A



B

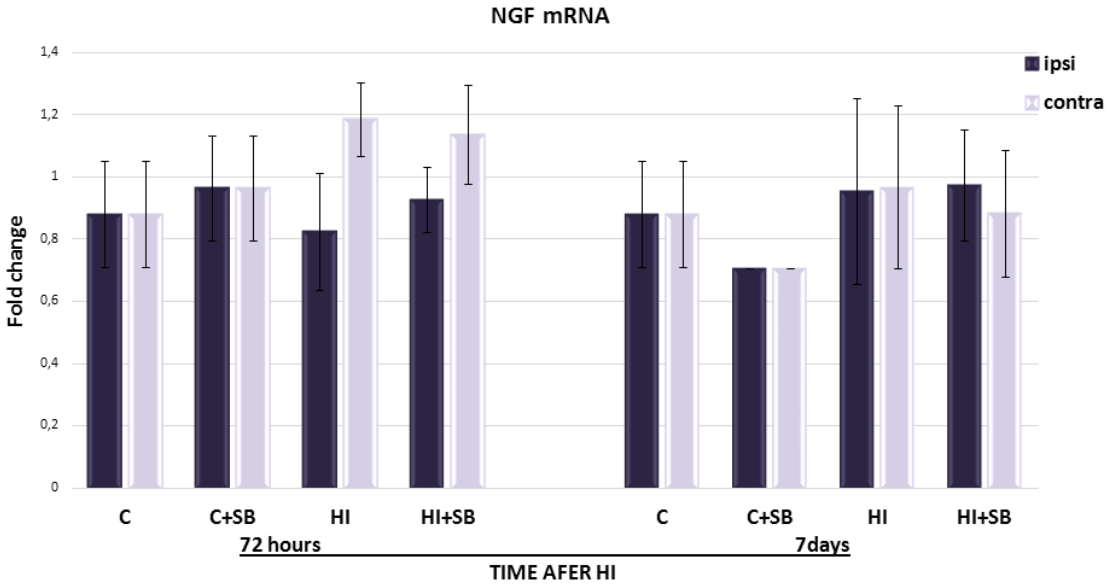


Fig. 27. Effect of sodium butyrate on neurotrophin gene expression in the brain after neonatal hypoxia-ischemia. (A) BDNF mRNA expression 72 hours and 7 days after HI. (B) NGF mRNA expression 72 hours and 7 days after HI. The fold change of the relative mRNA expression of each studied gene was calculated with the $2^{-\Delta\Delta CT}$ method. Data represent the normalized target gene amount relative to control which is considered to be 1. The values are means ± SD from 5 animals per group and time point assessed in triplicates. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, **p<0.01 ipsilateral HI vs. ipsilateral HI+SB. C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.7. Effect of sodium butyrate on neurotrophin receptors

Due to the fact that the biological effects of neurotrophins are mediated through binding to their receptors in the next stage the expression of specific for BDNF receptor – TrkB and its phosphorylated form, as well as low affinity receptor p75 were evaluated at different time points after HI.

4.7.1. Effect of sodium butyrate on TrkB receptor

To investigate whether the SB-induced changes in BDNF are accompanied by alterations in its receptor, the expression of total TrkB and its phosphorylated form (phospho-TrkB) was assessed after HI and drug treatment. The levels of protein were detected by evaluating immunoreactive band densities at 145 kDa. All data were compared to control at each investigated time point. A statistically significant effect of SB was observed only in the case of total TrkB receptor assay in the ipsilateral hemisphere at 7 days of recovery, when its expression demonstrated a value about two-folds higher than in control ($p<0.05$) (Fig. 28A).

Next the immunoreactivity of the phosphorylated form (active) of the receptor was determined. The analysis of the densitometric data revealed variable values, probably due to the high rate of phosphorylation/dephosphorylation reactions (Fig. 28B). HI injury at 24 hours of recovery led to a reduced expression of the phosphorylated receptor in the contralateral (hypoxic only) hemisphere, to about 65% of value characteristic for control ($p<0.01$). SB administration after the insult declined the immunoreactivity of phospho-TrkB in the ipsilateral side ($p<0.01$ ipsilateral vs ipsilateral with SB) and as a result, the expression of the phosphorylated form became equal in both hemispheres. Furthermore, changes in the level of phospho-TrkB were also observed after 7 days. The expression of phospho-TrkB in both hemispheres of HI animals decreased to 50% of the respective control ($p<0.001$). After SB treatment the level of the phosphorylated receptor returned to control value in the ipsilateral side ($p<0.001$ ipsilateral vs ipsilateral with SB). It is worth to point out that at this recovery time the reduction of phospho-TrkB after HI coincides with the level of total TrkB protein. Similarly, a correlation, although not as strongly pronounced, can be observed between phospho-TrkB and total TrkB in the ipsilateral hemisphere after SB treatment, when an increase and return to control value is noted.

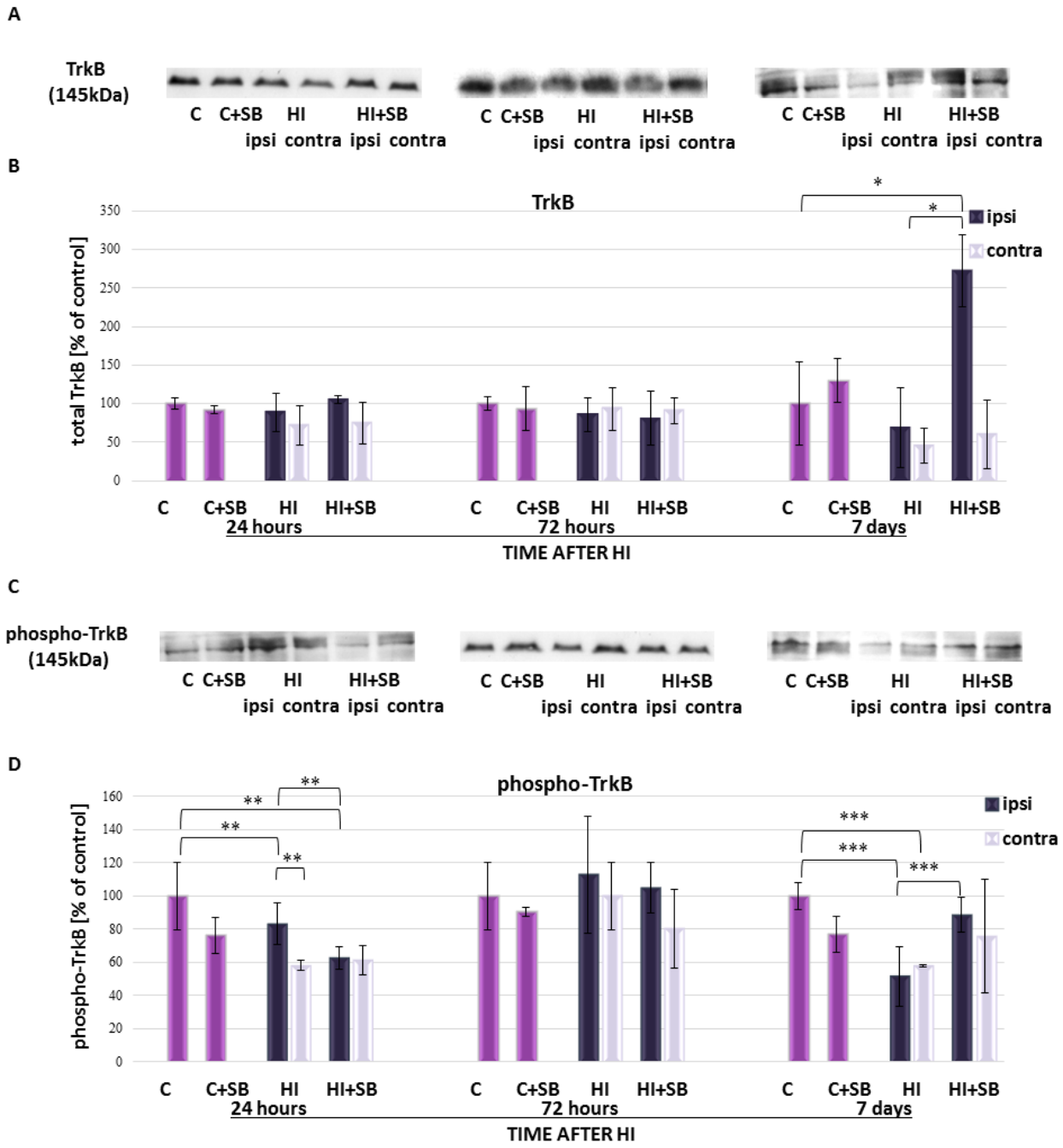


Fig.28. Effect of sodium butyrate on the expression of TrkB and phospho-TrkB in the brain after neonatal hypoxia-ischemia. Representative immunoblots of total TrkB (A) and phospho-TrkB (C) protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graphs (B and D) represent statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.7.2. Effect of sodium butyrate on receptor p75^{NTR}

Finally, the influence of SB on the expression of p75 receptor was checked. Brain tissue samples were blotted with corresponding antibody and the estimated band immunoreactivities are presented as a percentage of respective control. The effect of the insult alone (without SB treatment) was seen after 7 days and was exhibited by an increase in p75 immunoreactivity, almost two-folds when compared to control value. After SB treatment all values returned to sham level ($p<0.01$ ipsilateral vs ipsilateral with SB). It is worth to note that the effect of SB was also seen at earlier time of recovery with a significant decrease (by about 20%) in p75 protein expression in the ipsilateral hemisphere 72 hours after the injury ($p<0.05$ ipsilateral vs ipsilateral with SB) (Fig. 29).

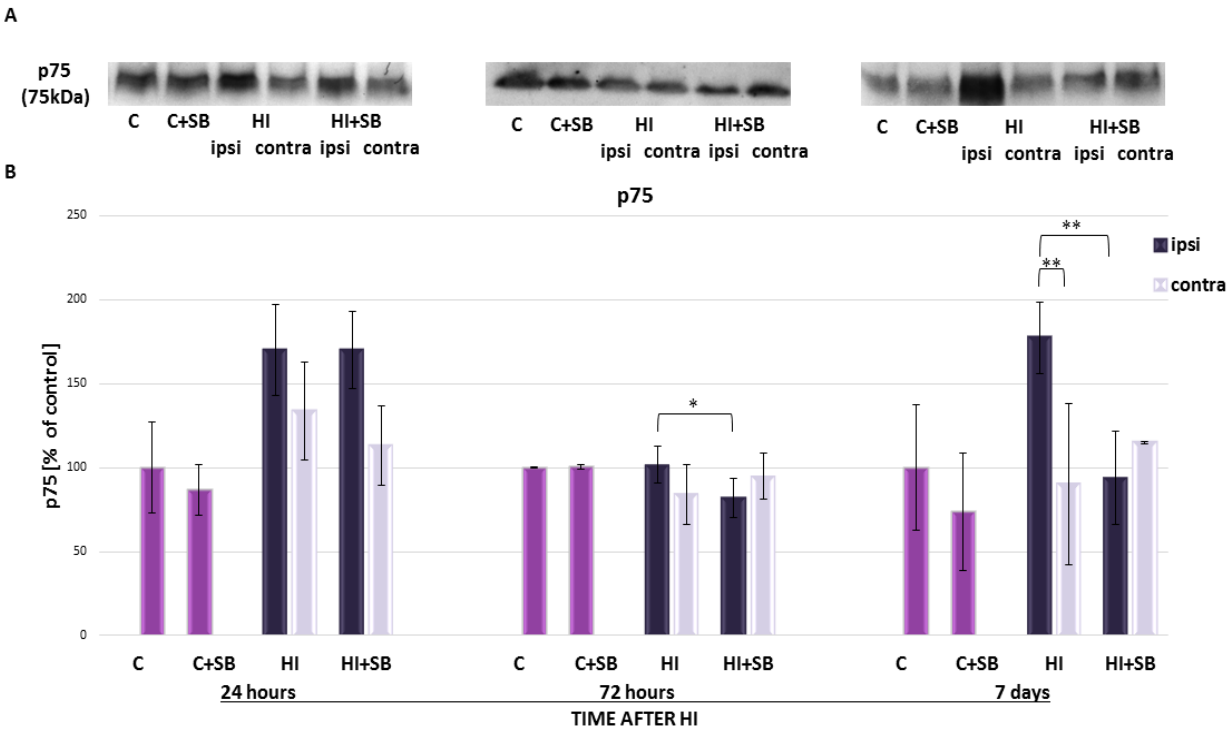


Fig.29. Effect of sodium butyrate on the expression of p75 in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of p75 protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05 and **p<0.01. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.8. The effect of sodium butyrate on the expression of protein kinases - ERK and Akt

Binding of neutrophins to their receptors initiate a signalling cascade that promotes cell survival through activation of Ras-MAP and PI3K /Akt pathways. Therefore, to evaluate whether the changes in neutrophins are accompanied by alterations in their downstream effectors, the expression of ERK and Akt kinases, as well as their active phosphorylated forms, were investigated.

4.8.1. Protein kinase ERK

The ERK and phospho-ERK levels were detected by evaluating the band densities at 42/44 kDa. Figure 30 shows representative immunoblots probed with antibodies specific to total ERK1/2 protein, as well as to phospho-ERK1/2, together with the densitometric analysis. Due to insufficient separation of samples, the data from two bands presenting ERK, as well as phospho-ERK (kDa 42/44), were summarized and presented as a percent of the respective control. As indicated in Fig 30A, the total ERK expression demonstrated close to control values in the analysed experimental groups during the entire course of this study. However, detailed analysis allows to state that one week post-insult there is, although a very subtle, tendency towards elevation of ERK expression in the damaged hemisphere after HI as well as after SB treatment.

Furthermore, the only change in phospho-ERK activity was noticed 7 days after the insult in the ipsilateral hemisphere (Fig. 30B). An increase after HI injury alone ($p<0.001$ ipsilateral HI vs. control), as well as, however less pronounced, in the group subjected to SB treatment was observed ($p<0.01$ ipsilateral HI with SB vs. control). Therefore, it may be suspected that at this time point the phosphorylation status might be related to the amount of total kinase protein.

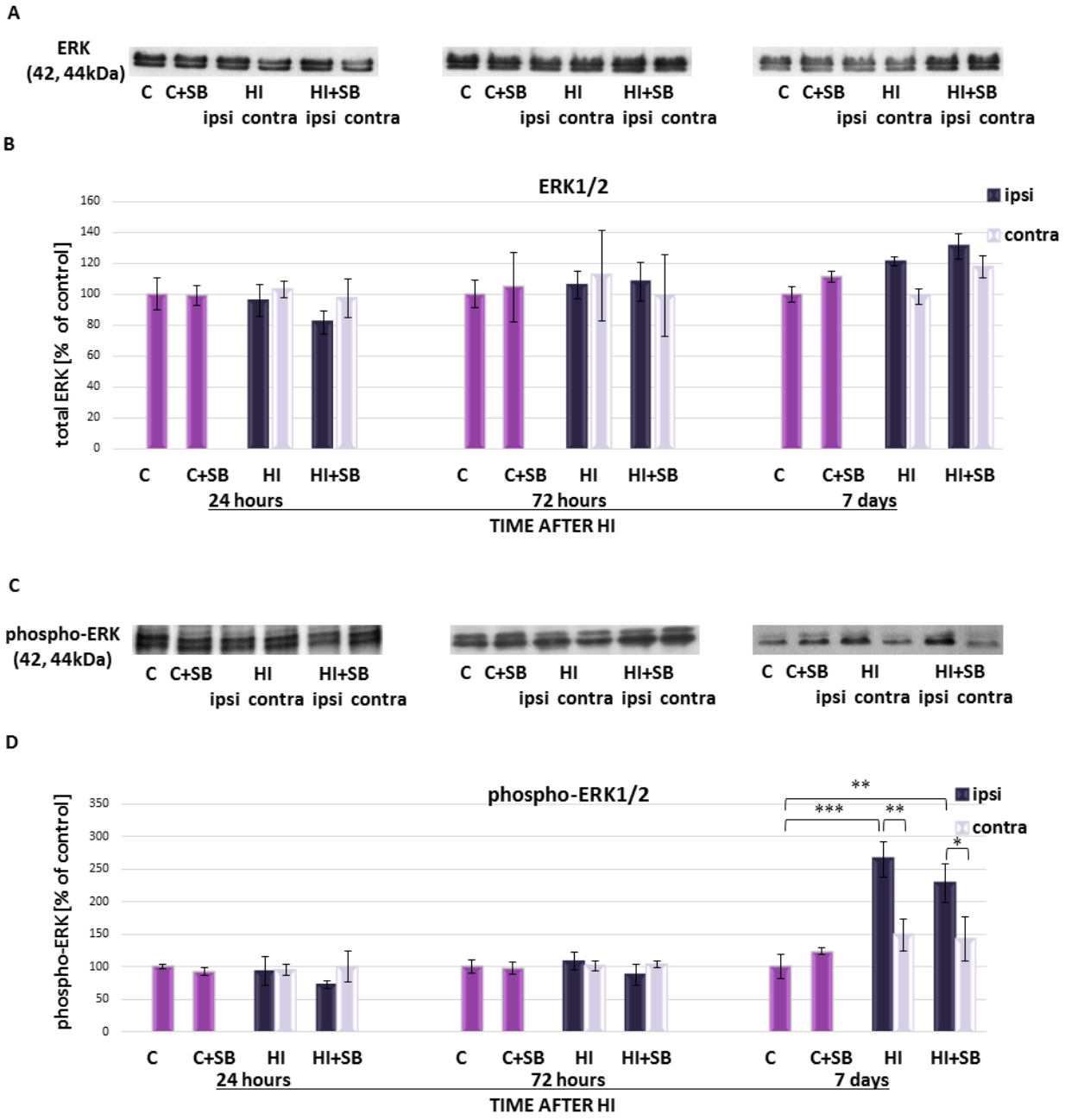


Fig.30. Effect of sodium butyrate on the expression of ERK1/2 and phospho-ERK1/2 in the brain after neonatal hypoxia-ischemia. Representative immunoblots of total ERK1/2 (A) and phospho-ERK1/2 (C) protein.The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graphs (B and D) represent statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.8.2. Protein kinase AKT

During the entire course of the experiment the amount of total Akt (Fig. 31A,B), similarly to total ERK protein (Fig. 30A,B), did not show significant changes. In contrast, the profile of Akt activity was temporarily different than that presented by ERK (compare C, D in Fig. 30 and Fig. 31). The data indicate a significant increase in phosphorylated Akt in the ipsilateral hemisphere by about 65% after HI, and more pronounced (by 90%) after SB treatment 24 hours post-insult ($p<0.0001$ control vs. ipsilateral HI and ipsilateral HI with SB) (Fig. 31 C, D). The observed enhancement of activity did not reflect the pattern of the total amount of kinase protein. Analysis of the respective blots did not show changes in the phosphorylated status of this kinase in comparison to the control during the prolongation of recovery time.

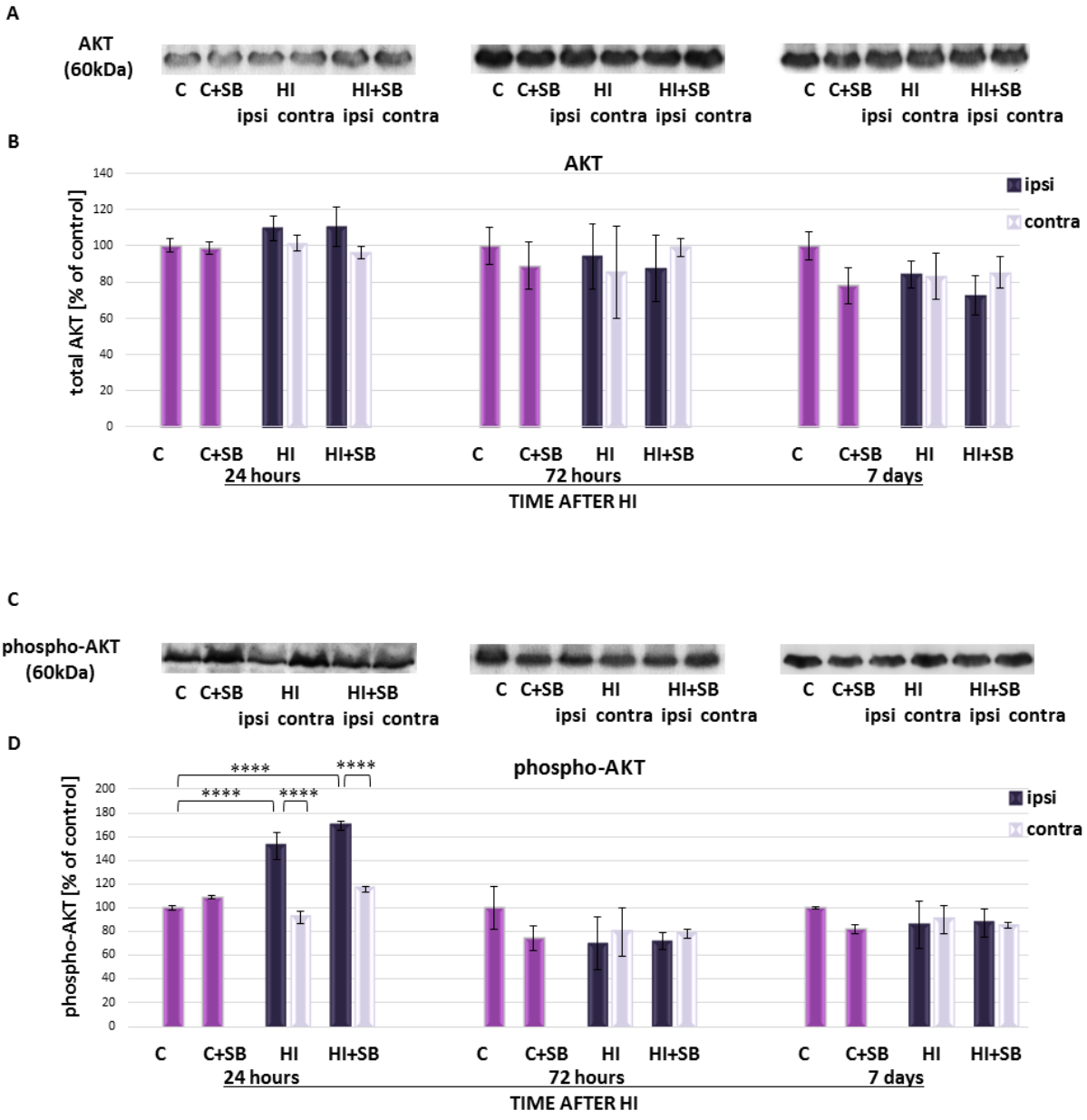


Fig.31. Effect of sodium butyrate on the expression of Akt and phospho-Akt in the brain after neonatal hypoxia-ischemia. Representative immunoblots of total Akt (A) and phospho-Akt (C) protein.The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graphs (B and D) represent statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, ****p<0.0001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.9. The effect of sodium butyrate on transcription factors (phospho-CREB, NFκB, p53)

In the next step the effect of SB on chosen transcription factors (phospho-CREB, NF-κB, p53) was checked.

4.9.1. Phospho-CREB

First, the effect of sodium butyrate on the expression of phosphorylated CREB (phospho-CREB (Ser133)) was examined. Phospho-CREB is an important transcription factor downstream of BDNF-TrkB signaling pathway. The Western Blot assay data, followed by densitometric analysis, showed that one day after HI the response of phospho-CREB to the insult was associated with a marked decrease of protein immunoreactivity in the ipsilateral hemisphere compared to control ($p<0.05$) (Fig. 32). Administration of SB restored the expression level to sham value. At 7 days after the injury the expression of phospho-CREB in the HI side increased to about 160% of the respective control and was not further influenced by HDACi treatment. In the contralateral hemisphere the immunoreactivity of this factor presented close to control values during the entire course of the experiment.

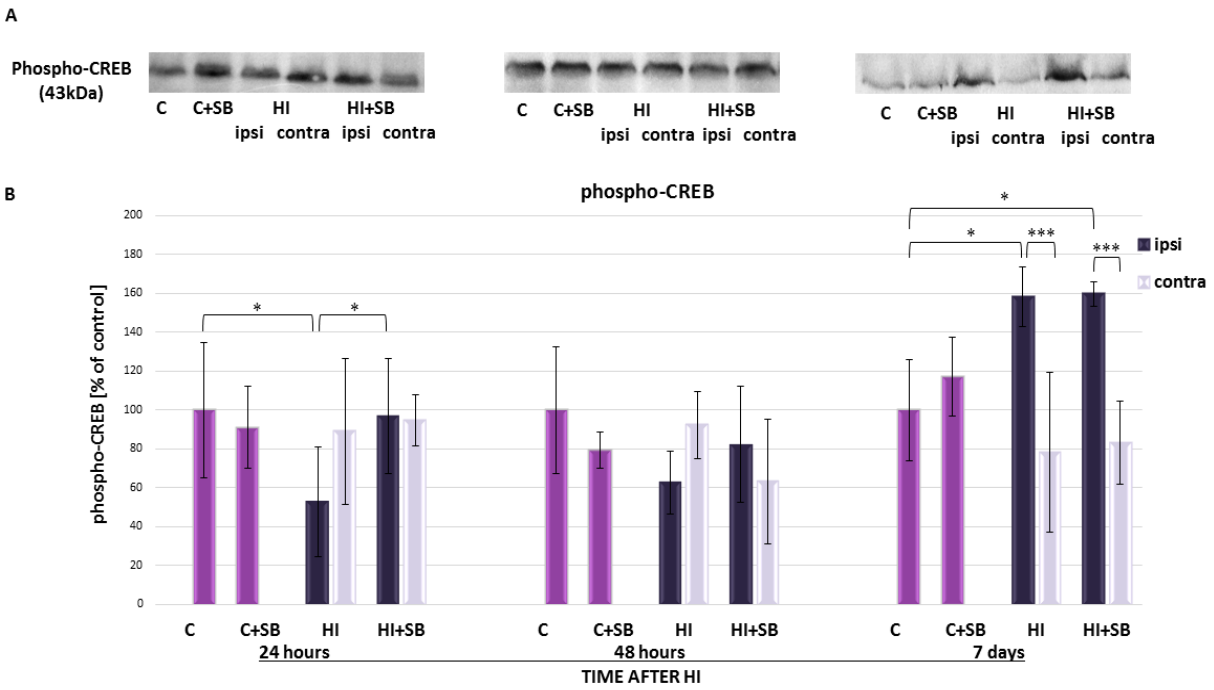


Fig.32. Effect of sodium butyrate on the expression of phospho-CREB in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of phospho-CREB protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.9.2. NF-κB

The next analyzed transcription factor was NF-κB. As shown in Fig. 33 exposure of 7-day pups to HI caused significant elevation of NF-κB, almost equally in both brain hemispheres (ipsi- and contralateral), compared to the sham control (about 1.5-fold; $p<0.01$) at 24 hours of recovery. As a result of SB treatment the level of protein returned to the control level and this was the only noticeable effect of the histone deacetylase inhibitor action. At 48 hours after the injury the level of NF-κB had the tendency to increase in both sides after the injury, however densitometric analysis of the respective blots did not indicate any significant changes between experimental groups. One week after HI the level of this protein remained close to control in all animal groups.

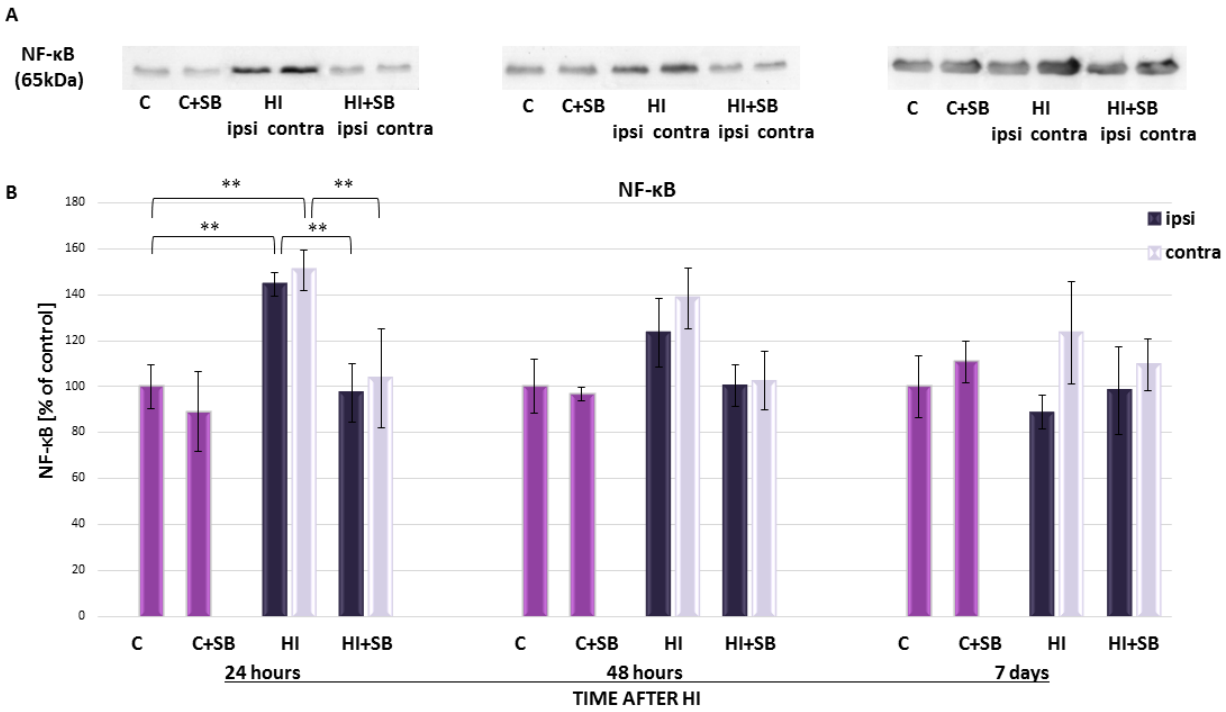


Fig.33. Effect of sodium butyrate on the expression of NF-κB in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of NF-κB protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, **p<0.01. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.9.3. p53

The level of p53, the apoptosis regulating transcription factor was estimated at 24, 48 hours and 7 days of recovery after HI. As shown in Figure 34, HI did not alter the immunoreactivity level during the course of the present study; however the trend was noticed towards higher expression of p53 in the hypoxic-ischemic hemisphere at 24 and 48 hours after the insult, compared with the hypoxic only, contralateral side, as well as with sham control. Importantly, administration of SB after the onset of HI in any case did not suppress the expression level of p53.

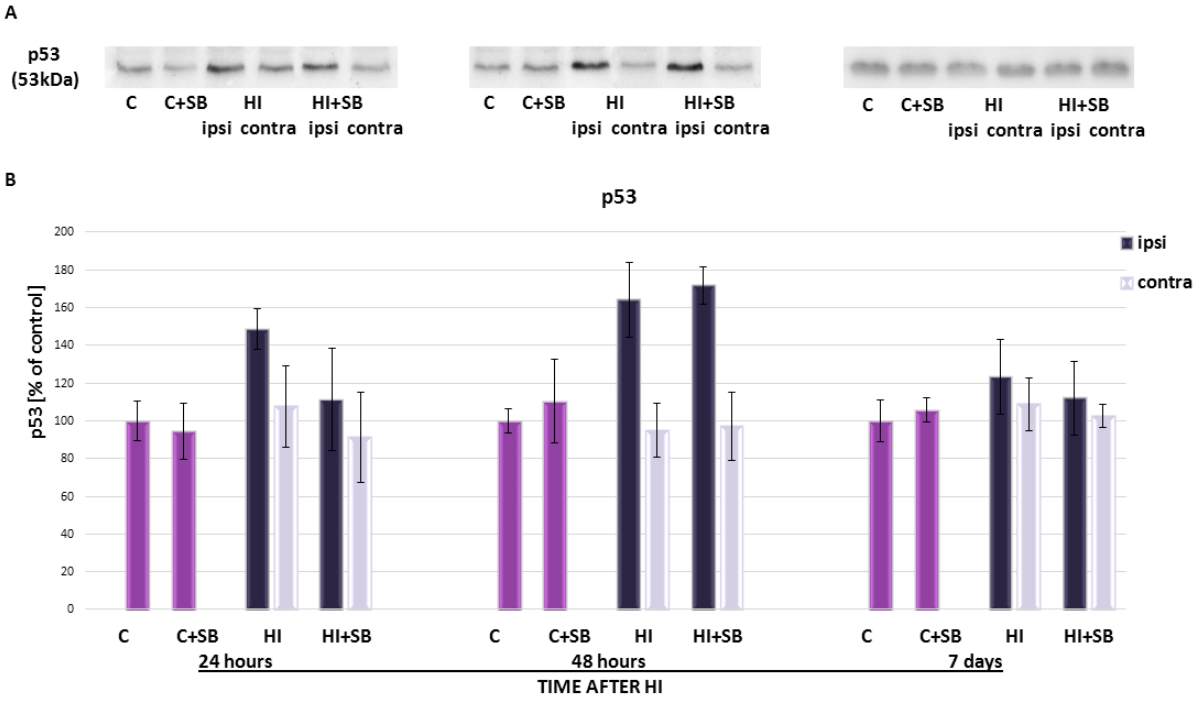


Fig.34. Effect of sodium butyrate on the expression of p53 in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of p53 protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test did not indicate significant differences between experimental groups. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.10. The effect of sodium butyrate on HSP70

According to generally accepted data indicating the correlation between HSP induction and resistance to brain damage, the role of HSP70 as a potential mediator of the neuroprotective effects of exogenously administered SB was evaluated. Figure 35 shows representative immunoblots and relative intensity of changes (quantified by scanning densitometry). The data revealed that 24 hours after HI, the immunoreactivity of HSP70 declined in the ipsilateral side to 80% of control values ($p<0.01$, control vs ipsilateral). No change in HSP70 expression in this time point in the contralateral hypoxic hemisphere of HI-treated rats was observed. Unexpectedly, the administration of SB led to a further decrease of HSP70 expression (to 69% of control; $p<0.001$). An increased expression of HSP70 after SB injection was found in both brain hemispheres, compared to respective vehicle-treated animals, at 7 days of recovery ($p<0.05$, vehicle treated vs SB treated).

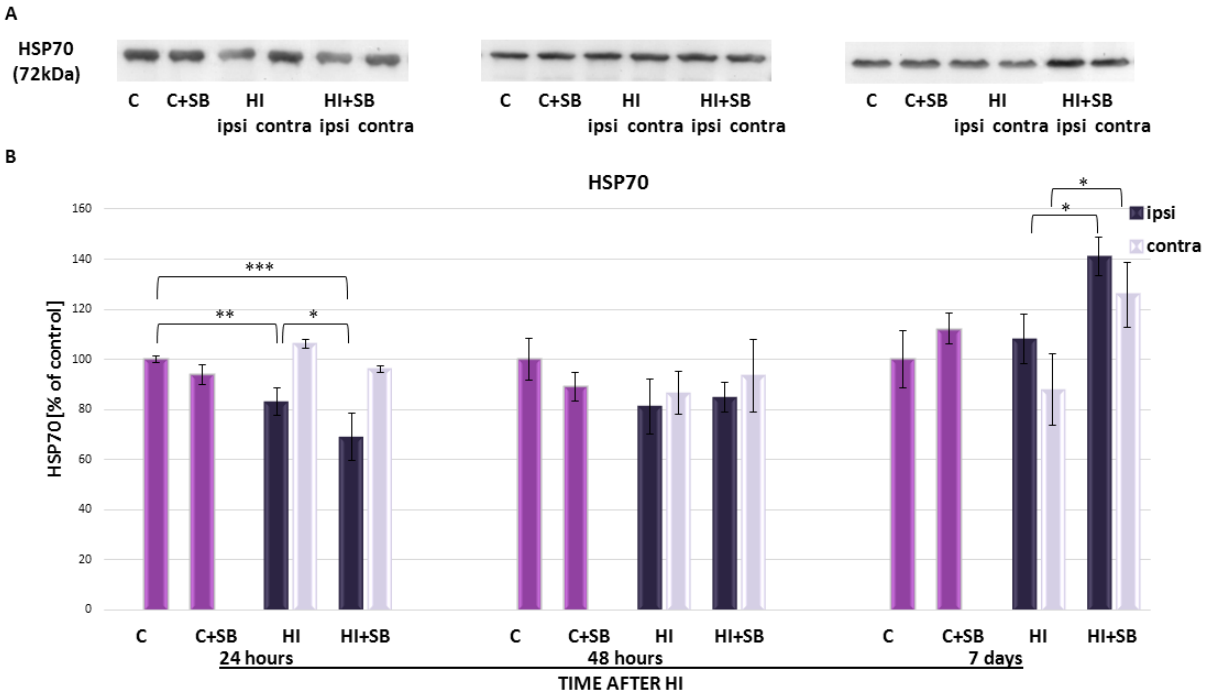


Fig.35. Effect of sodium butyrate on the expression of HSP70 in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of HSP70 protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.11. Effect of sodium butyrate on pro- and anti-apoptotic proteins

To address the question whether the neuroprotective/neurogenic action of SB is associated with an influence on apoptosis related factors the levels of activated caspase-3, Bax and Bcl-2 were assessed.

4.11.1. Caspase 3

Activity of caspase-3 is expressed by the level of fluorescence generated upon cleavage of specific caspase-3 substrate (N-Ac-DEVD-N'-R110). As shown in Fig 36, HI induced a significant increase in caspase-3 activity in the ipsilateral hemisphere noticed at 24 and 48 hours of recovery, compared to sham control ($p<0.0001$ and $p<0.05$, respectively). Only a tendency to increase the activity of caspase-3 was simultaneously observed within the contralateral hypoxic hemisphere at 24 hours after the insult. There was no effect of the HDACi - SB on the activity of caspase-3 in the injured ipsilateral side.

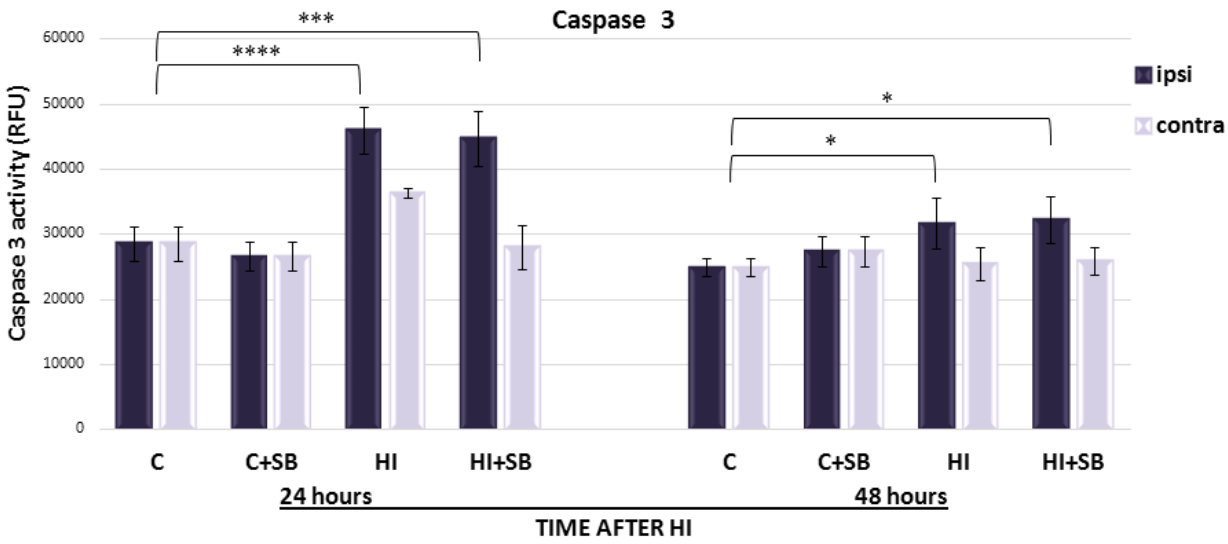


Fig.36. Effect of sodium butyrate on the expression of caspase-3 activity in the brain after neonatal hypoxia-ischemia. Bar graph represents statistical analysis of caspase-3 activity presented in Relative Fluorescence Units (RFU). The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05, ***p<0.001 and ****p<0.0001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.11.2. The assay of Bax and Bcl-2

The levels of pro- and anti-apoptotic proteins Bax and Bcl-2, respectively, were evaluated by Western immunoblotting technique. The analyses revealed that 24 hours after HI the level of pro-apoptotic protein - Bax increased in the ipsilateral side about two-fold compared to sham ($p<0.001$). As depicted in Fig. 37 higher than the control level of Bax expression was also observed at 48 hours of recovery ($p<0.05$). In contrast, at the same time, the expression of Bax protein in the contralateral hemisphere remained unchanged. SB injection had no apparent effect on the enhanced level of this protein in the brain after the injury.

Hypoxia-ischemia significantly increased the level of anti-apoptotic protein - Bcl-2 in the contralateral, non-injured side pronounced at 48 hours of recovery, compared to HI hemisphere ($p<0.05$). No other changes were noticeable, regardless of animal group (Fig. 38).

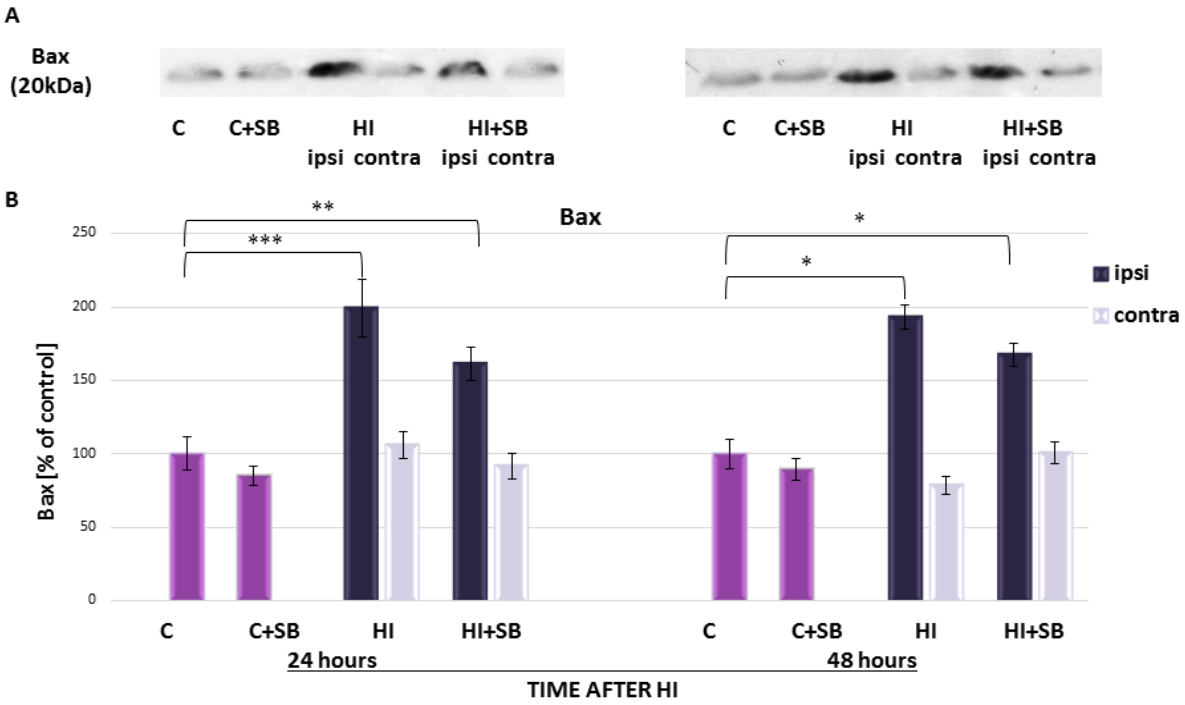


Fig.37. Effects of sodium butyrate on the expression of Bax in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of Bax protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

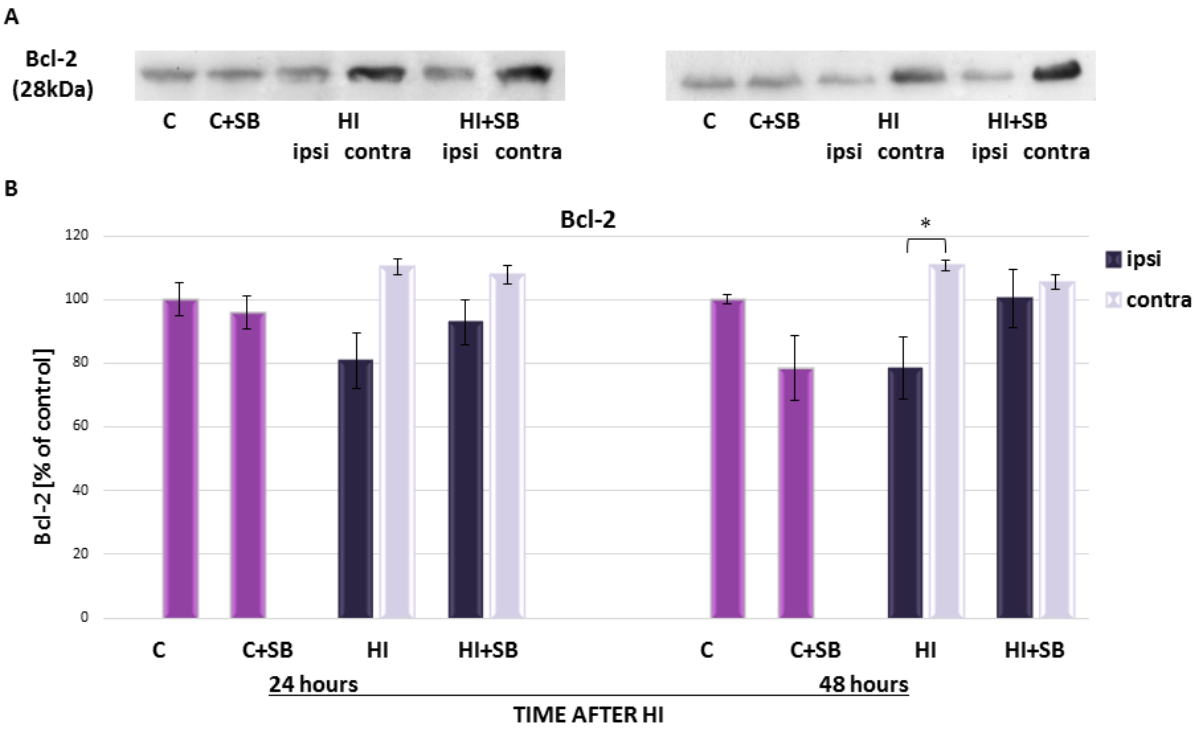


Fig.38. Effects of sodium butyrate on the expression of Bcl-2 in the brain after neonatal hypoxia-ischemia. (A) Representative immunoblots of Bcl-2 protein. The intensity of each band obtained by respective Western blotting was quantified and normalized in relation to β-actin. Bar graph (B) represents statistical analysis of densitometric data presented as percent of control value from indicated experimental groups. The values are means ± SD from 5 animals per group and time point assessed in 3 independent experiments. One-way ANOVA and Bonferroni test indicate significant differences between investigated groups, *p<0.05. Abbreviations: C *control*, ipsi *ipsilateral*, contra *contralateral*.

4.12. Behavioral testing

Finally, to evaluate the potential effect of SB treatment on functional autcome after HI injury, several behavioral tests were performed.

4.12.1. Open Field

On P33-34 the open field test was performed to assess the animals' response to a novel environment, their activity and locomotion abilities. There was an effect of the day $F(2,62)=7.3$, $p<0.01$, reflecting shortening of the distance travelled in consecutive days, but no effect of the group $F(2,31)=0.5$, $p=0.61$, and no group x day effect $F(4,62)=1.0$, $p=0.42$. Day differences were confirmed by post-hoc analysis in HI group (day 1 vs. day 3, $p<0.05$) and in HI+SB group (day 1 vs. day 3, $p<0.05$) (Fig. 39). Results of velocity paralleled these results. There were no other differences for duration and frequency in OF zones. Results of this experiment reflect that the administration of SB does not influence the response of animals to novelty or their locomotor skills.

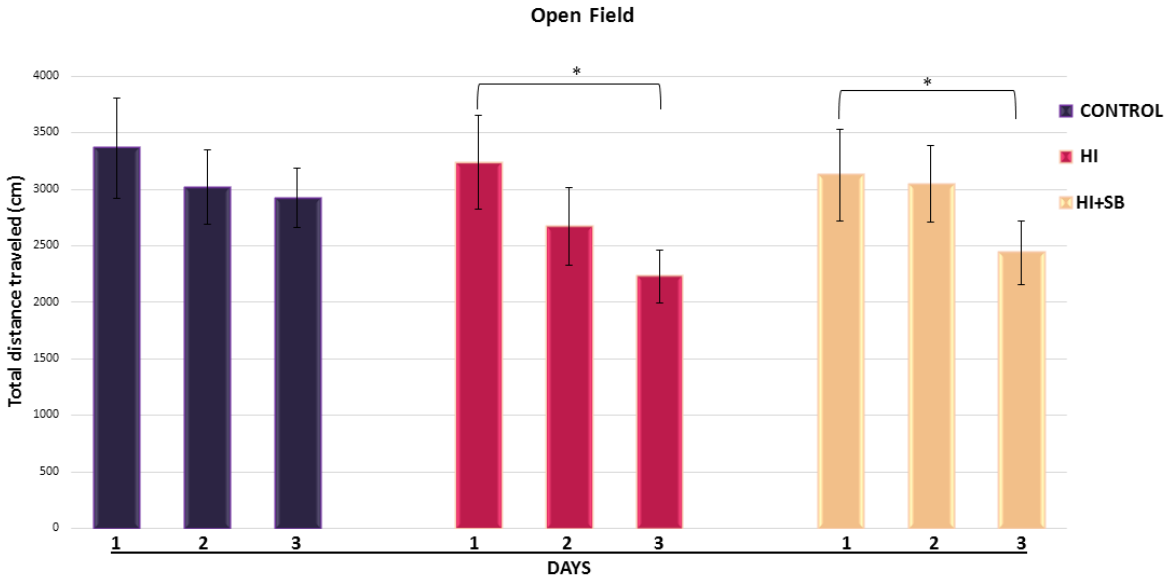


Fig.39. Effects of sodium butyrate on rat behavior in the Open field test after neonatal hypoxia-ischemia. Animal behavior was monitored on P33-34 in three investigated groups: control (C), hypoxia-ischemia (HI), and hypoxia-ischemia treated with SB (HI+SB). The scheme represents the data obtained by Open field test. The values are means ± SEM from 5 animals per group. One-way ANOVA and Duncan test indicate significant differences, *p<0.05.

4.12.2. Rotarod

On P39-40 the rotarod test was carried out to assess coordination, balance and gross motor functions. There was only an effect of the day, $F(3,60)=3.4$, $p<0.05$, reflecting general tendency for the animals to stay on the rod longer from day to day, with no group effect $F(2,20)=2.1$, $p=0.15$. However, a direct comparison (one-way ANOVA) of the first day results between sham control (165.1±15.2 N) and HI animals (124.1±12.1 N) ($F(1,14)=4.1$, $p=0.06$) and between control and HI+SB rats (101.4±14.0 N) ($F(1,19)=9.3$, $p<0.01$) suggest that the latency to fall was reduced in HI rats compared to age-matched controls (Fig. 40).

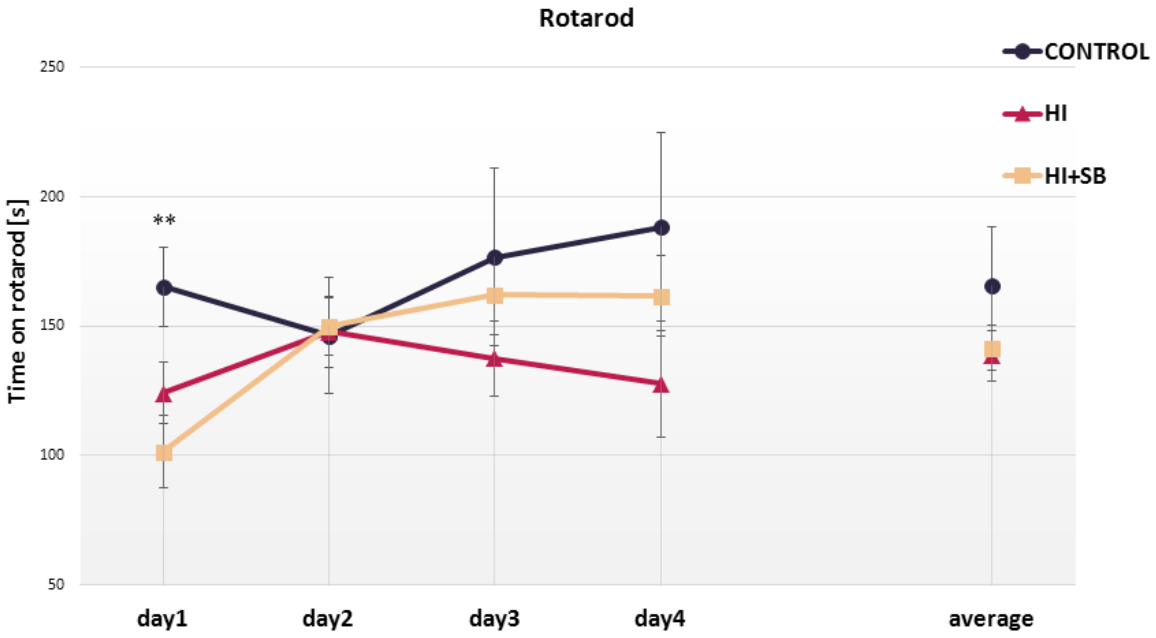


Fig.40. Effects of sodium butyrate on rat behavior in the Rotarod test after neonatal hypoxia-ischemia. Animal behavior was monitored on P39-40 in three investigated groups: control (C), hypoxia-ischemia (HI), and hypoxia-ischemia treated with SB (HI+SB). The scheme represents the data obtained by Rotarod test. The values are means ± SEM from 5 animals per group. One-way ANOVA and Duncan test indicate significant differences, **p<0.01.

4.12.3. Grip test

To quantify the impact of HI and SB administration on the muscular strength of animals the grip test starting from day P46-47 was used. There was no group ($F(2,32)=1.5$, $p=0.24$), and no day ($F(1,32)=0.1$, $p=0.74$) effect, illustrating that neither HI nor SB affected the grip strength of forelimbs (Fig.41).

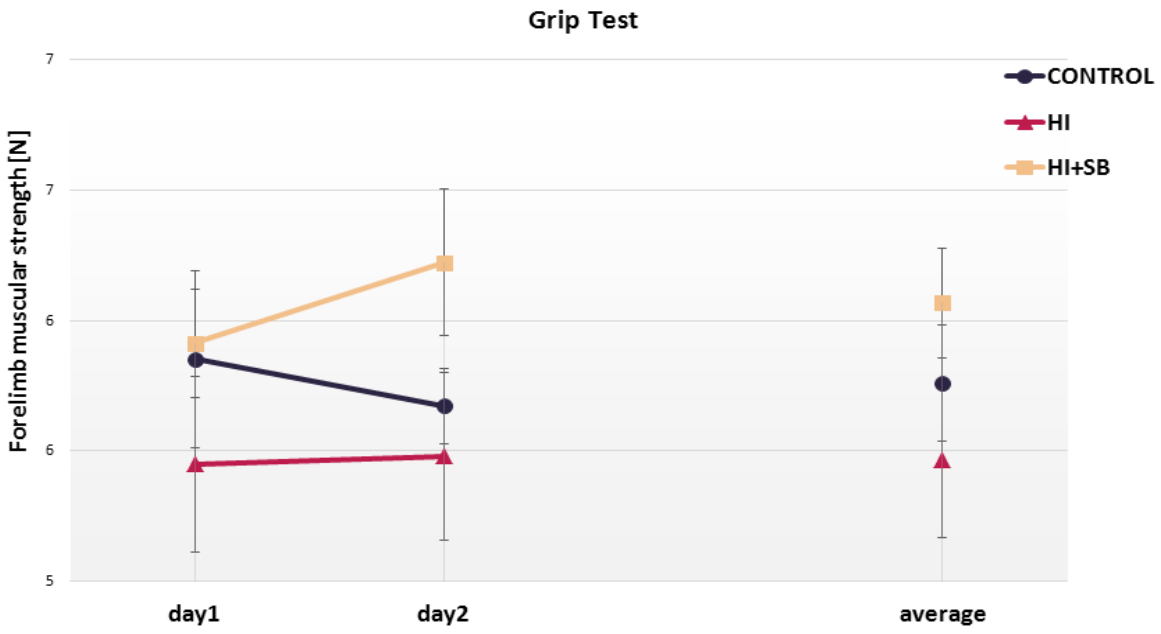


Fig.41. Effects of sodium butyrate on rat behavior in the Grip test after neonatal hypoxia-ischemia. Animal behavior was monitored on P46-47 in three investigated groups: control (C), hypoxia-ischemia (HI), and hypoxia-ischemia treated with SB (HI+SB). The scheme represents the data obtained by Grip test. The values are means ± SEM from 5 animals per group. One-way ANOVA and Duncan test did not indicate significant differences in animal behavior between experimental groups.

4.12.4. Morris Water Maze

Spatial learning and memory were assessed in animals starting from P62 using the Morris Water Maze test. Longer latencies to reach the platform in hypoxia-ischemia rats were noticed when compared to control animals, while SB-treatment had no major influence. After several days of learning, all animals showed shorter latencies in reaching the platform, with a strong effect of the day $F(5,160)=9.3$, $p<0.001$. There was also a general effect of the group $F(2,32)=3.8$, $p<0.05$ as well as a group x day effect $F(10,160)=3.7$, $p<0.001$. Post-hoc analysis showed a decrease in latency to reach the platform in control rats ($p<0.001$) as well as in HI+SB group ($p<0.01$) and almost significant effect in HI rats ($p=0.08$). Also, towards the end of the training (days 4-6), there was a difference between control and HI rats on day 4 ($p<0.01$), day 5 ($p<0.05$) and day 6 ($p<0.05$), whereas there was no significant difference between control and HI+SB rats during these days ($p>0.05$, Duncan). However, the results of HI+SB animals closely paralleled these of HI rats toward the end of the training (Fig.42). Thus, results obtained from this test suggest that HI animals have a more impaired memory performance than those with SB administration when compared to control animals.

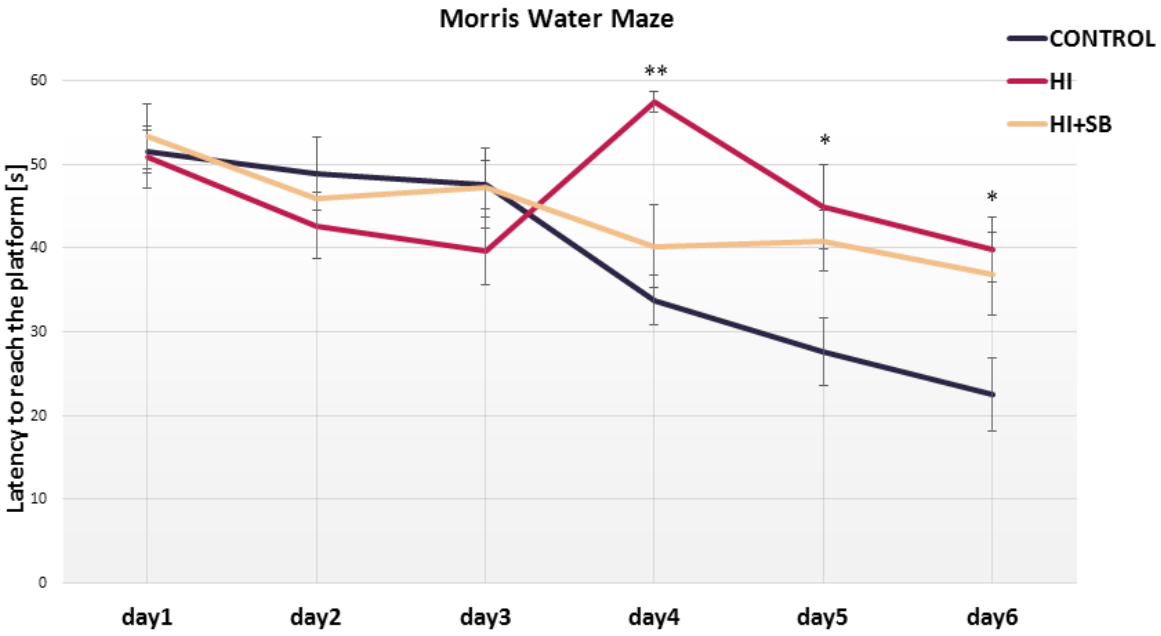


Fig.42. Effects of sodium butyrate on rat behavior in the Morris Water Maze test after neonatal hypoxia-ischemia. Animal behavior was monitored on P62-69 in three investigated groups: control (C), hypoxia-ischemia (HI), and hypoxia-ischemia treated with SB (HI+SB). The scheme represents the data obtained by MWM test. The values are means ± SEM from 5 animals per group. One-way ANOVA and Duncan test indicate significant differences, *p<0.05, **p<0.01.

4.12.5. *Ultrasonic vocalizations (USV)*

The evaluation of social and emotional behaviors was done by measuring the rates of 50 kHz USV. HI and HI+SB animals displayed less USV then control animals, especially when following the experimenters' hand. There was no significant effect of SB administration observed, i.e., no difference between HI and HI+SB groups. However the number of USV emitted by control animals was higher than in case of HI and HI+SB rats. This effect was especially visible during follow sessions. When average numbers of USV from all follow sessions were analyzed (ANOVA), there was the effect of group $F(2,14)=3.8$, $p<0.05$; with differences between control (40.0±3.5 USV) vs. HI (23.9±6.4) animals ($p<0.05$) as well as control vs. HI+SB rats (21.1±5.6) ($p<0.05$, Duncan). While there was no group effect for average USV emitted during play sessions, $F(2,14)=0.7$; $p=0.51$; control rats, 36.8±3.0; HI rats, 30.8±7.4; HI+SB rats, 28.3±3.4 USV) (Fig. 43). Results obtained in this task suggest that HI affected USV emissions at anatomical and/or behavioral levels.

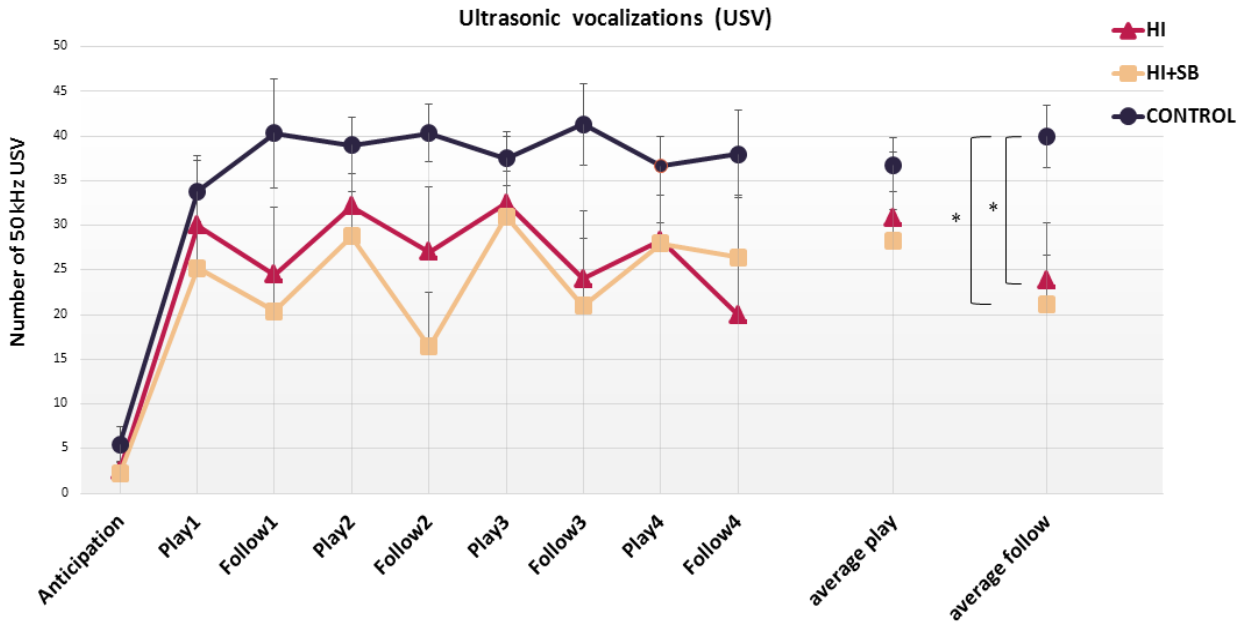


Fig.43. Effects of sodium butyrate on Ultrasonic vocalizations after neonatal hypoxia-ischemia. Animal behavior was monitored on P81 in three investigated groups: control (C), hypoxia-ischemia (HI), and hypoxia-ischemia treated with SB (HI+SB). The scheme represents Ultrasonic vocalizations of animals during the test. The values are means ± SEM from 5 animals per group. One-way ANOVA and Duncan test indicate significant differences, *p<0.05.

5. DISCUSSION

The present study showed that treatment with histone deacetylase inhibitor-sodium butyrate exhibits brain-protective activity in a neonatal hypoxia-ischemia model. The protection afforded by SB is expressed by a clear reduction of brain damage, suppression of brain edema (estimated at 6 and 14 days after the onset of hypoxia-ischemia), as well as increased neurogenesis. The striking features of these results are that neuroprotective/neurogenic effects of SB observed in this study are associated with expanded population of neuroblasts detected by staining with BrdU/DCX, expansion of oligodendrocyte precursor cells (BrdU/NG2-positive) as well as inhibition of HI-induced inflammation. However, unexpectedly, no significant improvement of the behavioral impairments seen in MWM and USV tests, which resulted from HI, was observed.

Neonatal hypoxia-ischemia sets in motion a series of pathophysiological processes (including loss of energy, acidosis, excitotoxicity, elevation of intracellular calcium, induction of oxidative stress, inflammation) that result ultimately in the massive loss of neurons and severe neurological deficits, despite the reported enhancement in the neurogenic response subsequent to neonatal brain injury. It is of note that many experimental studies have shown an increased number of proliferating neural progenitor cells residing in the active SVZ (Hayashi et al., 2005; Ong et al., 2005; Plane et al., 2004). Furthermore, these give rise to neuroblasts that according to some authors can migrate into the ischemia-damaged structure. However, in spite of these interesting and important findings, much doubt remains regarding the regenerative capacity because the expected long-lasting effect on neuron numbers was not noticed (Bartley et al., 2005; Hayashi et al., 2005; Yang and Levison, 2006). A few papers evaluating the proliferative capacity in the second neurogenic area-hippocampal SGZ, reported inconsistent data: either increased (Bartley et al., 2005) or reduced (Chang et al., 2006; Kadam et al., 2008; Yang and Levison, 2006) total counts of new cells. These obvious discrepancies could be explained by differences in experimental conditions including, among others, different strains of rodents, severity of the insult, and BrdU injection protocols, which undoubtedly contribute to the variability in the number of BrdU-positive cells among animals. During the course of this study, a BrdU administration protocol (4– 6 days after lesioning) was selected on the basis of experimental results as well as on the results of adult rodent stroke studies (Parent et al., 2002). It was anticipated that this paradigm would be optimal for labeling newly generated cells and that it would minimize the possibility of BrdU incorporation associated with DNA repair (Cooper-Kuhn and Kuhn, 2002).

Quantitative analysis of BrdU labeling in tissue samples gained 2 and 4 weeks after the injury shows that the population of proliferating cells in the damaged ipsilateral as well as in contralateral DG and SVZ is close to age-matched sham-operated animals with only the transient decrease of

marker incorporation 9 days after HI in both hemispheres in the SVZ. This observation is consistent with data reported previously by Qiu et al. (2007) in mice subjected to HI. Thus, it may be supposed that the immature brain is already working at the top of its proliferative capacity in this stage of development. It is also probable that the pool of progenitors might be preferentially protected against ischemic depletion.

The unchanged intensity of proliferation found in this study remains in striking contrast with a marked suppression of the population of new neuroblasts (BrdU/DCX positive cells) as well as newly matured granule neurons (BrdU/calbindin-positive) in the HI-injured ipsilateral side of the hippocampus. Therefore, it may be concluded that contrary to what has been assumed previously, the degree of endogenous spontaneous neurogenesis observed in dentate gyrus is insufficient for replacing the lost neurons and to achieve global repair of the neonatal brain injured by HI.

In contrast to the described above events noted in the neurogenic hippocampal area, hypoxic ischemic insult did not affect the pool of BrdU /DCX positive cells present within the SVZ area. Moreover, even the number of BrdU/NeuN labeled cells remained close to the sham control. The lack of response against HI indicates that the SVZ has been spared histologically by the injury, as was reported previously by Hayashi (Hayashi et al., 2005) and Scheepen (Scheepens et al., 2003). In contrast, a body of evidence indicates that hypoxic-ischemic injury stimulates an expansion of the ipsilateral SVZ in rodents (mice and rats) as a result of increased proliferation and leads to the increase in BrdU/DCX double positive cells number (Alagappan et al., 2009; Ong et al., 2005; Plane et al., 2004; Yang and Levison, 2006). In the course of the present study, the size of the SVZ remained unchanged, however adjusted the shape to the enlarged lateral ventricles. This discrepancy is possibly due to the differences in experimental conditions including strains of rodents, which may affect the extent of brain damage resulting from HI.

Furthermore, in contrast to others (Felling et al., 2006; Ong et al., 2005; Plane et al., 2004; Yang and Levison, 2006) no direct evidence for the streaming of new neuroblasts from the immature SVZ toward the postischemic striatum was found. Nevertheless, the current results demonstrate a small but sustained proportion of BrdU/NeuN labelled cells in this structure. In this situation, the possibility that new striatal neurons originate from local neural progenitors, possibly detached from the SVZ during maturation, and proliferate due to molecular changes in the cell environment after injury cannot be excluded. This hypothesis remains in agreement with the work showing ischemia-induced striatal neurogenesis in adult stroke model (Parent et al., 2002), despite that local progenitor populations are minimal. Recently performed cell fate studies (Duan et al., 2015) provide evidence that striatal resident reactive astrocytes can transdifferentiate into functional mature neurons in the adult mammalian brain *iv vivo* in response to ischemic injury, which is consistent with reprogramming striatal astrocytes *in vitro* (Magnusson et al., 2014). In addition, it was demonstrated

that activation of inflammation, the key pathogenic factor in ischemia, promotes a conversion of GFAP-expressing astrocytes *in vivo* toward the more immature state, characterized by re-expression of stemness markers and the repression of typical astrocyte markers (Gabel et al., 2016). It is supposed that cerebral injury could stimulate the activation of endogenous neurogenic transcription factors in the brain to direct reactive astrocytes to transdifferentiate to neurons, which does not occur in intact adult brains. Finally, the possibility is considered that NG2 positive cells in the normal developing or pathologic brain are also involved in the genesis of neurons (Yokoyama et al., 2006). However, it is worth to point that cell lineage commitment in the striatum was essentially not neuronal (Kadam et al., 2008). A substantial increase in GFAP-positive cells indicates that proliferating cells tend to differentiate toward astrocytic fate. This is supported by all studies in rodents independent of the strain used. It is therefore obvious that further research is needed to elucidate the generation of neurons in the striatum area.

The present study demonstrates that despite the severity of brain pathology induced by neonatal HI, administration of sodium butyrate for 5 days following the onset of the insult appears to decrease the cerebral damage by preventing severe atrophy or brain asymmetry. These findings are in general agreement with those reported previously that histone deacetylase inhibitors (VPA, TSA and SB) exhibit a neuroprotective effect in cerebral injury induced in adult rodents (Kim et al., 2007a, 2009; Ren et al., 2004). Thus, based on the beneficial effects proven in the above studies, the potential use of HDACis for the mobilization of endogenous progenitors seemed to be reasonable as a therapeutic strategy for restoring the damaged neonatal brain. Yet, only a few papers have presented a neuroprotective action of these agents in the immature brain and those addressing the impact of HDAC inhibitors upon post-injury neurogenesis remain particularly limited (Fleiss et al., 2012; George et al., 2013; Kabakus et al., 2005; Sandner et al., 2011). Moreover, the aims of the above mentioned studies did not always include the assessment of maturation of the proliferating cells into neurons and other cell types.

It is assumed that the reduced deficit of weight in the hypoxic-ischemic hemisphere after SB administration reflects compensatory formation of new cells. Surprisingly, it occurred that SB did not enhance the population of dividing cells in both neurogenic areas - DG as well as SVZ. As was mentioned above, it may be due to a maximal rate of cell proliferation in the chosen stage of brain development and then, it cannot be upregulated any further by epigenetic manipulation. Otherwise, exposure to SB restored the lost neuroblasts in the ipsilateral SGZ and, in addition, stimulated their generation in the ipsilateral SVZ 14 days after HI. This finding raises the possibility that new cells will participate in the recovery by supplementing new neurons. This prediction was reinforced by the fact that neuroblasts express DCX, a marker with multiple beneficial functions including migration, differentiation, and survival. However, the number of newly generated cells positive for both signals,

BrdU and calbindin, present throughout the DG granular cell layer, did not return to the control level, despite the apparently robust production of immature neurons after exposure to SB. In the same experimental paradigm, sodium butyrate enhanced the number of BrdU/NeuN positive cells in the ipsilateral SVZ. However this change was not as robust as observed in double labeled BrdU/DCX cells and was not significant compared to the control SVZ. This may indicate that a subset of DCX positive cells after the transient phase of up-regulation at 14 days post-HI do not survive process of maturation. It is consistent with previous data suggesting that most newly generated neuronal precursors derived from SVZ neurogenesis die before becoming mature cells (Arvidsson et al., 2002; Plane et al., 2004; Thored et al., 2006).

At present, a clear picture of the critical initial signaling events responsible for the insufficient neurogenesis in the presence of SB remains elusive. One of the most likely explanations is that the injured SGZ does not provide an environment that is conducive to the maturation or survival of the newly formed neurons, which is in agreement with other published data (Arvidsson et al., 2002; Plane et al., 2004; Snyder and Park, 2002). In line with this, growing evidence shows that HI insult alters the cellular and molecular composition of the neurovascular niche (Madri, 2009; Pathania et al., 2010; van Velthoven et al., 2011). Therefore, although cell proliferation is maintained in the neurogenic zones, detrimental changes in protein expression in the brain can lead to impairments in neuronal fate commitment and survival. It is supposed that an anti-neurogenic environment counteracts the development of neuroblasts and may contribute to the predominant glial fate *in vivo*, much like in the adult brain (Seidenfaden et al., 2006). The results of the detailed elegant study performed by George et al. (2013), indicates that the impact of HDAC inhibitors upon post-stroke neurogenesis is likely to depend on many factors, including the age of the animal at the time where neurogenesis is assayed, duration of HDAC inhibition before the BrdU labeling, and/or stage of the evolution of injury. Nevertheless, the results obtained in this work remain in disagreement with reports indicating that SB, as well as other inhibitor treatments, induced neuronal cell differentiation in brain areas injured by ischemia and may contribute to long-term beneficial effects in adult rodents (Kim et al., 2009; Ren et al., 2004).

Neonatal hypoxia-ischemia led to a marked reduction in the number of oligodendrocyte progenitor cells (OPCs; BrdU/NG2), pronounced in the ipsilateral hippocampus. This response remains in agreement with already published data showing particular vulnerability of oligodendrocytes progenitor cells to neonatal HI injury (Hayashi et al., 2005; Levison et al., 2001; Liu et al., 2002) and that they represent the main cell type responsible for the white matter damage seen after HI (Back et al., 2002). This contention was mainly based on results showing an increased vulnerability of oligodendrocyte progenitors to glutamate, free radicals, as well as inflammatory products (Back et al., 2002). OPC depletion may lead to impairment of oligodendrocyte maturation at

a premyelinating developmental stage and in consequence to white matter damage (Rezaie and Dean, 2002).

However, the markers attributed to more advanced stages of oligodendrocyte maturation (BrdU/O4-positive cells) seem to be similarly distributed in control and injured animals at 4 weeks after HI. It might be due to ongoing compensation by the active gliogenesis process which is known to proceed most intensely during both the perinatal period and the first postnatal weeks in rats (Gallo and Deneen, 2014; Rowitch and Kriegstein, 2010; Semple et al., 2013). Since OPCs are supposed to be generated in excess during ontogenesis, the number of mature oligodendrocytes is often reported to be normal in spite of their decreased number after insult (Kucharova and Stallcup, 2015; Trapp et al., 1997). These processes remain in line with only slight decreasing tendency in the number of NG2/BrdU labeled cells in ipsilateral SVZ and unchanged number of O4/BrdU cells in this area after HI. This implies the protection of myelin formation which is still in active state in neonates (Zaidi et al., 2004).

Post-insult treatment with SB-histone deacetylase inhibitor robustly increased the number of OPCs in both hippocampi at 2 weeks of recovery reinstating the physiological pool of OPCs, which could contribute to sustain endogenous homeostasis. The present data are consistent with findings from *in vitro* studies pointing to the role of SAHA, another HDACi, in preserving mature oligodendrocytes in the mouse optic nerve after OGD (Baltan et al., 2011). On the other hand, Fleiss et al. (2012) using a model of lipopolysaccharide-sensitized hypoxia-ischemia found that trichostatin A, which is also a common inhibitor of HDAC, did not affect the insult-reduced oligodendrocyte number, what could be observed by Olig-2 staining. The obvious differences could be due to different investigation models. It is worth to point out, that there are also interesting but intriguing data indicating that just histone deacetylase activity is essential for oligodendrocyte differentiation in the developing rodent brain (Jacob et al., 2011; Marin-Husstege et al., 2002; Ye et al., 2009). Of note is that activity of class I histone deacetylases (HDAC1 and HDAC2) is required to regulate oligodendrocyte differentiation and maturation by reducing the expression of co-repressors of myelin gene transcription (Liu and Casaccia, 2010; Ye et al., 2009). Furthermore, genetic ablation of both isoforms in the mouse blocks these processes (Ye et al., 2009). However, it seems rather hard to compare the study conducted by Ye et al. in non-pathological experimental conditions with hypoxic-ischemic brain injury performed in this study. Therefore, considering this data the role of HDAC inhibition in mediating the effect observed in the course of this investigation cannot be negated.

One of the interesting findings obtained in the current work is that the protective effect of SB on oligodendrocyte progenitors seen in the ipsilateral hemisphere 2 weeks after the insult was associated with reduction of HI-induced microglial cells and infiltration of macrophage/monocytes (ED1-positive), most important pathogenic components of neonatal brain damage. It might be one of

the potential mechanisms whereby SB protects oligodendrocytes. This is generally consistent with reported earlier correlation between oligodendrocyte protection and suppression of pro-inflammatory action of microglia/macrophages in adult rodents (Kannan et al., 2013; Kim and Chuang, 2014; Liu et al., 2012).

Convincing evidence reveals that HDACis, among them VPA, TSA, and SB, are efficacious neuroprotective agents and treatment in adult cerebral injury, after the onset of stroke, results in a marked reduction of microglia number, suppression of their activation, and inhibition of other inflammatory markers, which in turn leads to improved neuropathological outcome (Kim et al., 2007a, 2009). As demonstrated in the current study, SB robustly diminished the generation of microglial cells (BrdU/ED1 positive) in the ipsilateral hemisphere. Furthermore, the majority of ED-1+ cells present a positive reaction with an established marker of M2 microglia phenotype, arginase-1, mostly pronounced in the SB-treated rats. The neuroprotective effect of SB expressed by regulation of the microglial inflammatory response via downregulating the expression of pro-inflammatory mediators and upregulating anti-inflammatory factors was confirmed recently in an experimental mouse model of MCAO (Patnala et al., 2016). In the light of these findings it may be speculated that SB facilitates conversion of M1 to M2 phenotype leading to anti-inflammatory signaling and, by this, keeps microglia from acquiring a pro-inflammatory phenotype, and in consequence prevents tissue damage, such as that found in models of AD, MS, and neurodegeneration (Cipriani et al., 2011; Fuhrmann et al., 2010; Koning et al., 2007). This prediction may be reinforced by the parallel decrease in the number of ED1/IL-1β-positive cells observed in the present study. Transition of microglia during recovery from the pro-inflammatory (M1) to immunomodulatory and neurotrophic response (M2) (Bonestroo et al., 2013; Shrivastava et al., 2013) and then maintenance of endogenous neurogenesis (Aarum et al., 2003; Hu et al., 2015; Starossom et al., 2012) may play a key role in attenuation of brain damage (Cikla et al., 2016). To confirm the role of microglial reaction to HI injury in the developing brain and, in particular, to define the time course of M1 to M2 polarization, further studies will be needed.

The reactive astrocytosis also appears to be a part of the hypoxia-ischemia-induced pathological processes (Sen and Levison, 2006; Sullivan et al., 2010). Consistent with previous reports (Bona et al., 1999; Burtrum and Silverstein, 1994; Shrivastava et al., 2013), an increase in GFAP expression accompanied with hypertrophy in the damaged ipsilateral hemisphere was noticed 14 days after the insult, implying astrogliosis. The role of astrogliosis after HI remains controversial, especially with respect to the beneficial or detrimental influence on CNS recovery. These glial cells synthesize and release several growth factors to enhance neurogenesis (Gengatharan et al., 2016; Sultan et al., 2015). However, they also express several inhibitory molecules that block or reduce adult neurogenesis and accelerate the formation of glial scars in the injured brain (Boda and Buffo,

2010). On the other hand, experimental disruption of astroglial scar formation following stroke results in an increased spread of inflammation and increased lesion volume (Li et al., 2008). Such dichotomy remains unresolved especially in the immature brain. Sodium butyrate treatment after HI insult further markedly increased GFAP expression. Based on our previous data presenting a reduced population of astrocytes expressing IL-1β after HI and SB treatment a beneficial action of astroglial cells can be suggested (Jaworska et al., 2017). Moreover, at the same time point, the report showed that SB application also diminished the IL-1β production in microglia/macrophages. The reduction of IL-1β expression after SB injection in glial cells paralleled the attenuation of brain damage. The precise molecular mechanism responsible for the effect of SB is not known. Apart from the number of biochemical and morphological factors functioning in concert to influence the final effect of the inhibitor, accumulation of GFAP protein presented here is likely to also contribute to neuroprotection after neonatal HI. This may be supported by data showing that GFAP knock-out mice have a greater susceptibility to ischemic injury (Nawashiro et al., 2000). Although results obtained from adult animal experiments cannot be directly transferred and used as explanation for neonatal data due to differences in the level of maturation and different ischemia models, some hypotheses may be valid in adults as well as in neonates. Therefore, the precise role of enhanced astrogliosis seen after SB treatment in neonatal HI is yet to be determined and efforts should be directed to improve the neurogenesis capacity, which could in turn enhance brain repair after a HI insult.

At present, it is rather impossible to precisely outline the molecular mechanisms directly linked to the observed beneficial effects of sodium butyrate after neonatal HI. One, most probable scenario, is the increased acetylation of histones which is a virtue of HDAC inhibitory activity. Indeed, HDACi treatment has been reported to be associated with marked upregulation of Acetyl-H3 in the DG after ischemia in adult rodents (Kim et al., 2007a; Ren et al., 2004). In our study SB application elicited an increase in acetylated histone H3 level only in sham control animals. In contrast to others, the same mode of SB application after hypoxia-ischemia did not result in the elevation of histone acetylation above the vehicle control value. Unchanged acetylation after HDACi injection was also described by Fleiss et al. (2012) in male mice in a model of LPS/HI. The unexpected lack of HDACi effect could be due to stress response of post-injured tissue which temporarily induced inhibition of histone acetyltransferase activity and/or over-activation of HDACs during this stage of development. This prediction may be reinforced by data reported by Sandner et al. (2011) showing prolonged (lasting 4 months) over-activation of HDAC after hippocampal lesioning. If such an event took place during the time of this assay it may account, at least partially, for the lack of SB influence. On the other hand, it is also possible that the evolving inflammatory response to HI injury may temporarily suppress HDAC activity producing a lack of response to inhibitors (Faustino et al., 2011). Nevertheless, the current results, independent of the above considered mechanism, may reflect a

novel equilibrium reached between acetylation and deacetylation processes in order to maintain the proper gene transcription.

Based on the present results, It could be stated that the effects of sodium butyrate treatment do not seem to be achieved through increased acetylation of histone H3. Histones are not the sole substrate of HDACs. Several of histone deacetylase isoforms have been shown to regulate acetylation of a plethora of non-histone proteins, and thus, the possibility that the neuroprotective effect of HDAC inhibitors are multifold and linked to influencing a diverse array of targets cannot be ruled out (Langley et al., 2009; Marks, 2010).

One of the HDACi targets known to be involved in neurogenesis and survival of newborn cells after ischemic insult is BDNF (Benraiss et al., 2001; Pencea et al., 2001). HDAC inhibition activates BDNF promoter IV and increases BDNF mRNA levels in dissociated rat cortical neurons (Yasuda et al., 2009). In line with this, application of HDACi after neonatal HI protected against the decreasing level of mRNA encoding BDNF. Furthermore, the increased level of precursor, pro-BDNF, and mature BDNF as well as NGF in the ipsilateral hemisphere found in this study after SB treatment may imply that trophic support plays a role in maintaining neurogenesis in the developing brain. This hypothesis has been intensified by reports showing that administration of BDNF after HI in neonates decreased brain damage and provided neuroprotection notably by facilitating anti-apoptotic signaling (Almli et al., 2000; Han and Holtzman, 2000). Nerve Growth Factor also appears to be neuroprotective in a neonatal model of HI (Holtzman et al., 1996). It was found that ICV administration of NGF or BDNF increased the number of new neurons and neuronal precursors generated in the neurogenic zones – SVZ and SGZ of adult brains and protected against neuronal death (Calza et al., 2003; Chiaretti et al., 2008; Zigova et al., 1998). The effect of BDNF and NGF appears to be mediated by competitive signaling between their specific high-affinity receptors TrkB and TrkA, respectively, and low affinity receptor $p75^{NTR}$ (Davey and Davies, 1998) . Treatment with SB prevented reduction of BDNF level caused by HI in the ipsilateral side and led further to a simultaneous increase of the TrkB protein amount. Such coincidence might suggest that Trk-BDNF signaling may play a role in the observed increased number of BrdU/DCX positive cells. The engagement of this pathway in mediating HDACi-induced cell proliferation and differentiation in ischemic adult rodents has been previously confirmed by Kim and Chuang (2014) and Kim et al. (2009). Thus, at least, this mechanism mediated by BDNF underpinning neuroprotection after SB treatment in neonates is supposed to be one of those commonly reported in adult cerebral studies. In addition, SB application also prevented the HI-induced reduction of NGF protein. The beneficial action of NGF enhancement by pretreatment with SB was reported in other brain injuries (Suzuki-Mizushima et al., 2002; Valvassori et al., 2014; Varela et al., 2015). It is suggested, that the survival response of nerve growth factor is mediated by TrkA receptor, which activation may be associated with the suppression of JNK kinase activity, the

mediator of hypoxia-induced neuronal cell death (Davis, 2000). Nevertheless, a probable criticism of the present study is that it was not possible to detect the level of TrkA; however the alteration of NGF in the presence of HDACi could imply that this response participates in neuroprotection.

In contrast to the diminished level of TrkB receptor in the damaged HI hemisphere, $p75^{NTR}$ increased significantly and its amount returned to control after SB treatment. A similar reaction of p75receptor in the presence of HDACi was described in a model of TBI (Lu et al., 2013b). The mechanism that induces p75 expression during injury still remains to be defined. More recently, the pro-inflammatory cytokines highly expressed in HI may correlate with p75 upregulation (Choi and Friedman, 2014; Jaworska et al., 2017).

The present findings raise the issue of the significance of $p75^{NTR}$ action. Several published data point out to a controversial role of p75 receptor in regulating neuronal fate, specifically in potentiating the effects of Trk receptors aimed at neuronal survival. It was also found that p75 deficient mice display reduction of adult neurogenesis (Young et al., 2007). On the other hand, numerous subsequent studies have highlighted the role of p75 overexpression in neuronal and glial cell death in adults after brain injury (Roux and Barker, 2002; Volosin et al., 2008). Thus, the biological importance of maintaining the p75 receptor in the HI hemisphere on the control level after SB treatment is not possible to define. Therefore, it seems reasonable to postulate that life and death decisions induced by neurotrophins appear to be governed by the level of expression and signaling activities of the p75 and Trk tyrosine kinase receptors and then activation and integration of downstream signaling molecules pathways.

Survival promoting effects of neurotrophins are elicited by activation of different intracellular signaling pathways. The first one includes the recruitment of phosphatidylinositol 3-kinase (PI3 kinase) and its downstream serine/threonine kinase Akt (protein kinase B) required for cell survival signaling by tyrosine kinase receptors. The other pathway involves the extracellular signal –regulated kinase (ERK) signaling, which further leads to cyclic AMP responsive element binding protein (CREB) phosphorylation. The data regarding the effect of experimental ischemia performed in adult animals on activated phospho-Akt are contradictory and show either a temporal increase of the enzyme level (Cheon et al., 2016; Endo et al., 2006; Li and Zuo, 2011) or a decrease (Kim et al., 2007b; Ouyang et al., 1999; Wei et al., 2017). Also the results of different HDACi treatments are not unified and either lead to the enhancement of Akt phosphorylation level (Kim et al., 2007b; Sinn et al., 2007; Wang et al., 2016) or , as in the case of another inhibitor, for example ITF2357, they do not influence Akt activity at all (Shein et al., 2009). The results presented in the dissertation showed increased immunoreactivity of active phosphorylated kinase Akt in the ipsilateral side at 24 hours of recovery with/or without SB treatment, which probably participates in the early short-term mobilization to protect against the injury. This suggestion remains in line with the view that PI3K/Akt seems to be

the principal transducer for pro-survival factors involved in trophic support. However, at present it is not possible to define precisely the mechanism responsible for the above patterns of changes.

The pathway involving Ras/Raf/mitogen-activated protein kinase /ERK kinase signaling cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression. In contrast to PI3/Akt, the response of ERK1/2 kinase to HI was demonstrated by a later enhancement of active phospho-ERK - at 7 days of recovery in the damaged hemisphere without further changes induced by HDACi treatment. These results fit to the assumption that in damaged cells ERK1/2 activation may act as an important sufficient defensive mechanism that could participate in mediating protective effects against injury in the adult and neonatal brain (Hetman and Gozdz, 2004; Jiang et al., 2015; Wang et al., 2003). The present results do not allow to state that the ERK activation after HI is directly linked to the TrkB-mediated singaling since the level of immunoreactivity of both proteins do not show a temporal relationship. However, such situation might take place after SB treatment, when the change in the activity level of TrkB reflects the change in phospho-ERK. Kinase ERK1/2 can promote cell survival by activation of both transcriptional–dependent as well as independent mechanisms, by inhibiting anti-apoptotic proteins (Bonni et al., 1999; Zhou et al., 2004).

Both ERK1/2 phosphorylation and its downstream target – transcription factor cAMP response element binding protein (CREB) have been implicated in the pro-neurogenic activity and neuroplasticity by inducing several target genes (Barnabé-Heider and Miller, 2003). The level of phospho-CREB reduced by HI in the ipsilateral hemisphere was reversed by SB treatment only 24 hours after the insult. This result parallels the work of others reporting up-regulated expression of phospho-CREB caused by SB or VPA treatment in different ischemic models (Kim et al., 2009; Sinn et al., 2007). Interestingly, a significant enhancement in phospho-CREB level was also observed at 7 days of recovery in the HI hemisphere and at this time point this change is observed regardless of SB treatment. The lack of further activation by SB may be realted to that presented by active ERK1/2. Thus, it may be postulated that the activation of phospho-CREB in response to HI may represent a cellular form of defense. Such speculation could be supported by previous work showing stimulation of CREB phosphorylation in adult ischemic animals (Hu et al., 1999; Mabuchi et al., 2001; Tanaka et al., 2000). However, the lack of the SB effect in this study 7 days after HI may be related to the sufficient level of activated CREB after the injury in the immature brain.

Several findings indicate that inhibitors of histone deacetylases may modify other than CREB non-histone targets such as NFκB , p53, proteins of the HSP family, and apoptosis-related genes (Bolden et al., 2006; Kim et al., 2007b; Spange et al., 2009). A number of reports point to the damaging role of activated by brain ischemia nuclear factor NFκB. This is supported by studies showing that inhibition of NFκB activation after ischemia in adult rodents prevents brain damage in

the insulted hemisphere via inhibition of cytokine response (Buchan et al., 2000; Kim et al., 2010). However, the current study revealed that following neonatal hypoxia-ischemia the expression of NFκB increased significantly in both hemispheres, ipsi- and contralateral at 24 hours after the insult, despite tissue alterations not being observed in the hypoxic, uninjured side. Moreover, in both hemispheres, the level of NFκB returned to the control value after SB treatment. Thus, the question arises whether the response of NFκB to SB may constitute part of the defense process against HI-induced damage in the ipsilateral side.

Another important molecule whose activity is induced after cellular stress is p53-apoptosis regulating transcription factor. The implication that p53 participates in the response that follows a hypoxic-ischemic insult stems from the observation that pifithrin-α, an inhibitor of p53 , decreases the number of apoptotic cells in the ischemic brain (Leker et al., 2004). In contrast to the robust upregulation of p53 detected in the adult ischemia model in rodents and inhibition of p53 protein levels by SB (Kim et al., 2007b), HI induced in neonates with/or without SB treatment did not show any significant effect. Thus, p53 seems not to be engaged in the events leading to neuronal cell death after neonatal hypoxia-ischemia.

An additional suggested factor by which HDACis are reported to mediate neuroprotection in adult cerebral injury models is HSP70 (Choi et al., 2014; Ren et al., 2004; Shein et al., 2009). HSP70 before functioning as a key member of the molecular chaperon system has been assigned an anti-apoptotic function, although failure to detect protection against apoptosis in neurons overexpressing HSP70 also has been reported (Mailhos et al., 1994). Nevertheless, most studies describe increased expression of HSP70 as a neuroprotective mechanism in adult rodents after MCAO (Faraco et al., 2006; Rajdev et al., 2000; Ren et al., 2004), as well as after neonatal HI (van den Tweel et al., 2006). The suggested influence of HSP70 action includes inhibition of nuclear transcription factor NFκB. In contrast to high expression of HSP70 at 12-48 hours found by van den Tweel et al. (2006) in the damaged HI hemisphere, the present results show significant reduction of this protein level at the same time point regardless of exposure to SB. Additionally, the changes in HSP70 expression observed in this study do not parallel alterations seen in the level of NFκB. The major difference with our study is that we used P7 vs P12 rats and a different time of hypoxia - 60 vs 90 min of hypoxia insult used by van den Tweel et al. (2006). The reason for the loss of HSP70 may be due to a low rate of its synthesis or increased activity of proteases able to digest HSP70. Also, our results are more clearly in agreement with Sun et al. (2015), showing that HSP70 is only slightly altered, if at all, in P7 neurons after HI. Interestingly, SB treatment caused elevation of HSP70 expression in both brain hemispheres 7 days post-HI. It seems that such delayed response detected in both hemispheres has to be insult independent. It may be also considered that induction of HSP70 after SB treatment may facilitate neuroplasticity during recovery time and improve learning processes (Lin et al., 2004).

Finally, the current results revealed no apparent effect of SB on components of programmed cell death pathway - activated caspase3, as well as expression of apoptotic protein Bax, which were significantly elevated after HI. There was also no expected effect on the level of anti-apoptotic protein Bcl-2. Therefore, these targets probably do not mediate SB-induced neuroprotection, in contrast to blockade of apoptotic mediators by HDACis in adult rodents subjected to ischemia (Faraco et al., 2006; Kim et al., 2007b; Lu et al., 2013b; Ren et al., 2004; Sinn et al., 2007). Therefore, the present findings show clearly that the considered above mechanism underpinning neuroprotection by SB in the developing brain is not any of those commonly reported in adult cerebral studies.

One would expect that the neuroprotective/neurogenic effects of SB found in this study after HI induced in neonatal rats would be further translated into improving the neurobehavioral performance. To this end sensorimotor as well as cognitive tests were employed in order to assess motor coordination and memory deficits in animals after HI, treated and untreated with SB. It was expected that by the time of testing (P33–P83), newly generated neuroblasts would become fully matured. In contrast, their number still remained below control level in the ipsilateral DG after HI as well as after SB treatment and only slightly higher in the injured SVZ. Only in two of the five individual tests performed (MWM and USV recording), HI-injured rats showed significant behavioral deficiency when compared to controls. Mildly impaired cognitive function observed in other tests performed indicate that HI-treated rats showed some degree of recovery as it has also been suggested by previous studies (Kiss et al., 2009; Lubics et al., 2005). Administration of the histone deacetylase inhibitor after the onset of the insult did not counteract significantly the functional brain impairments caused by HI; however, there was a tendency for improving learning, as is seen in the graph presenting the results of MWM. In spite that SB protected against ischemia-induced damage of brain structures contributing to several cognitive processes (Broersen, 2000; Rolls, 2000), the remaining number of new-generated mature neurons in the DG probably occurred to have insufficient potential for the compensation of interplay between hippocampus and prefrontal cortex. Thus, the rescue of neurobehavioral deficits needs restoration of the granule cell layer and preservation of the neural networks. Our data are similar to those reported by Sandner et al. (2011) showing that HDAC inhibitor injected for as long as 7 weeks was sufficient to alleviate some, but not all behavioral impairments due to neonatal ventral hippocampal lesioning. However, in the above-mentioned experiment, there was a clear discrepancy between injury progression and behavioral outcome. In contrast to the present findings, in adult rodent models of cerebral injury treated with HDACis, functional improvement correlated steadily with neurogenesis and reduced the severity of neurological injury (Kim et al., 2007b, 2009; Liu et al., 2012; Sinn et al., 2007). Consistent with these reports, many studies have shown that experimental reduction of adult neurogenesis impairs

hippocampal memory formation and conversely, stimulated neurogenesis seems to be one approach to enhance cognitive recovery. On the other hand, contradictory accounts showed no impact of adult brain neurogenesis reduction on memory formation, where the role of newly generated neurons has previously been strongly suggested (Hersman et al., 2015; Jaholkowski et al., 2009; Urbach et al., 2013). In the view of this inconsistent data, a stronger link between increased neurogenesis in the injured brain and functional outcome after treatment with HDACis needs to be pursued with future work.

6. SUMMARY OF RESULTS

1. Treatment with histone deacetylase inhibitor – sodium butyrate after neonatal HI reduces brain damage.
2. SB restores hypoxia-induced loss of neuroblasts and oligodendrocyte progenitors.
3. SB increases the number of mature neurons in the ipsilateral SVZ, whereas in the DG the number of mature granule neurons remains persistently lower than in control.
4. Administration of SB inhibits HI-induced inflammation, expressed by a decrease in microglia/macrophage accumulation within the injured tissue. The contribution of the increased astrogliosis in this process is yet to be determined.
5. SB prevents the HI-induced decrease in neurotrophins (BDNF and NGF), TrkB receptor an phospho-CREB. In contrast, SB significantly reduces the level of $p75^{NTR}$ which increases after HI injury.
6. None of the investigated molecular mediators that are known to be affected by HDACis in adult animals are apparently modified after SB administration (protein kinases ERK1/2 and AKT, pro- and anti-apoptotic proteins).
7. SB treatment does not improve behavioral outcome after HI.

7. CONCLUSIONS

In conclusion, this study provides evidence that treatment with HDACi – sodium butyrate has a neurogenic/neuroprotective effect in a neonatal hypoxia-ischemia injury model. Although it was not possible to completely determine the underlying neuroprotective mechanism, these results demonstrate that SB appears to exert a beneficial effect via suppression of HI-induced cerebral inflammation. Further studies will elucidate whether this HDAC inhibitor has a role in future clinically applicable neurotherapies.

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