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Research article

Stroke promotes survival of nearby transplanted neural stem cells by decreasing their activation of caspase 3 while not affecting their differentiation

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ABSTRACT

Although transplantation of stem cells improves recovery of the nervous tissue, little is known about the influence of different brain regions on transplanted cells. After we confirmed that cells with uniform differentiation potential can be generated in independent experiments, one million of neural stem cells isolated from B6.Cg-Tg (Thy1-YFP)16Jrs/J mouse embryos were transplanted into the brain 24 h after induction of stroke. The lateral ventricles, the corpus callosum and the striatum were tested. Two and four weeks after the transplantation, the cells transplanted in all three regions have been attracted to the ischemic core. The largest number of attracted cells has been observed after transplantation into the striatum. Their differentiation pattern and expression of neuroligin 1, SynCAM 1, postsynaptic density protein 95 and synapsin 1 followed the same pattern observed during *in vitro* cultivation and it did not differ among the tested regions. Differentiation pattern of the cells transplanted in the stroke-affected and healthy animals was the same. On the other hand, neural stem cells transplanted in the striatum of the animals affected by stroke exhibited significantly increased survival rates reaching 260 ± 19%, when compared to cells transplanted in their wild type controls. Surprisingly, improved survival two and four weeks after transplantation was not due to increased proliferation of the grafted cells and it was accompanied by decreased levels of activity of Casp3 (19.56 ± 3.1% in the stroke-affected vs. 30.14 ± 2.4% in healthy animals after four weeks). We assume that the decreased levels of Casp3 in cells transplanted near the ischemic region was linked to increased vasculogenesis, synaptogenesis, astrocytosis and axonogenesis detected in the host tissue affected by ischemia.

1. Introduction

Acute ischemic brain stroke is the second most common cause of death and far the most significant cause of permanent life-long disability [1]. With costs of care larger than one billion euros per year, ischemic stroke represents the most devastating medical problem of the humankind. The only existing treatment is fibrinolysis which has a very narrow therapeutic window of only few hours [2].

In the last two decades, a rather large number of both preclinical and clinical studies revealed that transplantation of stem cells promotes histological and functional recovery of the brain, including neurodegenerative and neurovascular diseases [3,4]. Tested cell types included embryonic stem cells, neural stem cells, mesenchymal stem cells and induced pluripotent stem cells [5]. Mechanisms of the observed beneficial effects of the transplanted cells include modulation of neuroinflammation, trophic effects and buffering of extracellular matrix. Several publications, including our own studies, have analyzed to which extent transplanted cells survived in the tissue affected by neuroinflammation. It has been shown that 50–70% of the cells die within one month after transplantation, which is linked to activation of caspase 3 and 6 [4,6,7].

Surprisingly, very few data exists on influence of the various host locations on the stem cell transplants, especially in regard to their survival and differentiation. Similarly, although the synapse represents one of the major features of neurons with a critical role in cell survival, very few data exist on influence of the host tissue on process of formation of synapse among transplanted cells.

To elucidate behavior of stem cells after transplantation into various anatomical locations in the brain affected by stroke and to test how stroke affects cell survival, synaptogenesis and differentiation, NSCs

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were transplanted into the lateral ventricles, corpus callosum and striatum of the mouse model of stroke. This study has shown that different brain regions influence transplanted NSCs, and this influence is differently displayed in stroke-affected animals and in their wild type controls. Surprisingly, stroke supports survival of NSCs in striatum by reducing activation of Casp3, but it has no influence on differentiation rate and development of synaptic contacts.

2. Material and methods

2.1. Animals and housing

B6.Cg-Tg(Thy1-YFP)16Jrs/J(Thy1 YFP-16) mouse strain (The JacksonLaboratory, Bar Harbor, ME, USA) was used for experimental purposes. The animals were kept on an standard 12/12 h light/dark cycle in a temperature controlled room (22 ± 2 °C, with 55% ± 10% humidity) at the animal facility at the Croatian Institute for Brain Research and were genotyped using the method published by our group [8]. Pelleted food and water were given *ad libitum* except during testing. All experiments on animals were approved by the Internal Review Board of the Ethical Committee of the School of Medicine University of Zagreb (04-77/2010-238) and Faculty of Veterinary Medicine (251/61-01/139-13-4), and were conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used forscientific purposes. The minimum number of laboratory animals nedeed was calcultated by power analysis which justifed the sample size used.

2.2. Isolation and cultivation of neural stem cells

NSCs were isolated from telencephalic wall of 14.5 days old embryos originating from Thy1-YFP mouse strain. Isolated stem cells were cultivated in a proliferation supporting medium comprising of DMEM/F-12 (Gibco by Life Technologies, ThermoFisher Scientific, Pittsburgh, PA), B-27 Supplement (Gibco by Life Technologies), N-2 Supplement (Gibco by Life Technologies), Penicillin/Streptomycin (Gibco by Life Technologies), Recombinant Mouse Fibroblast Growth Factor-basic (FGFb, ThermoFisher Scientific, Pittsburgh, PA, USA) and Recombinant Mouse Epidermal Growth Factor (EGF, ThermoFisher Scientific). Cells were cultivated in suspension, and after two days neurospheres were formed. For the purpose of differentiation analyses neurospheres were dissociated, single cells and small neurospheres were plated on 12-mm coverslips (200–250 000 cells/coverslip) as well as on 6-well plates (1 000 cells/well). Coverslips and wells were previously coated with Poly-D-lysine (500 µg/mL, 24 h at 37 °C, Sigma) and laminin (10 µg/mL, 24 h at 37 °C, Sigma). 24 h after plating, the medium was changed into the Neurobasal (Gibco by Life Technologies) which supports neurons *in vitro* and antibiotic with glutamine (Pen Strep Glutamine, Gibco by Life Technologies). Cells were differentiated in followed time points: 0, 1, 3, 5 and 7 days after plating.

2.3. Labelling of NSCs

Single NSCs were labelled with two exogenous dyes according to manufacturer instruction: some cells were labelled with PKH26 fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit for general cell membrane labelling, Sigma), while the other cells were incubated with 10 µM 5-Bromo-20-deoxyuridine (BrdU, Z99% (HPLC), Sigma) solution for eight hours.

2.4. Middle cerebral artery occlusion (MCAO)

Ischaemic brain injury was induced by transient left MCAO in three months old wild type mice which weighting 25–30 g. Operation was performed in inhalation anaesthesia, mixture of 2% isoflurane in 100% O_2. Surgery was performed under dissection microscope (Stemi DV4 Spot, Zeiss). During surgery, animal body temperature was maintained with heating pad. Following ventral neck surgery and blood vessels preparation, intraluminal filament (Doccol Company, Sharon, MA, USA) was inserted through common carotid artery (CCA) into the internal carotid artery (ICA) to the origin middle cerebral artery (MCA) and left for 90 min. A complete block of blood flow was confirmed by Doppler sonde. After 90 min intraluminal filament was withdrawn, perfusion was restored and animal subcutaneously received analgetic buprenorphine (0.03 mg/kg).

2.5. Magnet resonance imaging

High resolution *in vivo* MRI was performed on anaesthetized mice after MCAO by using 7.0T BioSpec 70/20 USR MRI system (Bruker BioSpin, Ettlingen, Germany) equipped with an 660 mT/m MRI RF gradient coils and 2-channel receive-only surface radiofrequency (RF) array coils (Bruker BioSpin, Germany) for mouse brain. Adult mice were anaesthetized with 4% isoflurane in a 70/30 nitrogen/oxygen gas mixture in the animal induction chamber. During MRI imaging, the anaesthesia was maintained with approximately 1.5% of isoflurane, and core body temperature and breathing rates were monitored by custom-made MobileLab Table (Medres, Germany) that contains complete setup for anaesthesia for small animals and includes temperature control unit and control system for measurment of physiological parameters (PhysioBox). T2 wted (T2W) images of stroke were aquired using a TurboRARE sequence. T2 mapping for T2 relaxation time calculation was done according to MSME sequence protocol (MSME_T2map, Bruker, Germany). Afterwards, an MRI imaging was performed in a following form: Diffusion Weighted Image Spin-Echo (DWI-SE) and Diffusion-Tensor imaging (EPI-DTI) using Echo-Planar imaging.

2.6. Stereotaxic injection of NSCs into the mouse brain transplantation (coordinates)

Previously labelled NSCs were re-suspended and injected in the final concentration of one million cells in 1 µL DMEM medium. For stereotaxic transplantation we used two groups of mice: healthy and stroke affected. Stereotaxic coordinates were determined according to stereotaxic atlas [9]. NSCs were injected into the three brain regions: lateral ventricle (AP −0.5, ML + 2 and DV −2.5), corpus callosum (AP −1.3, ML +2.0 and DV −1.5) and striatum (AP −0.5, ML + 2.5 and DV −2.5). Injections were performed using small animal stereotaxic apparatus (900LS, David Kopf Instruments, Tujunga, CA, USA) and Hamilton syringe needle 1 µL. Avertin (Sigma) was used for anaesthesia at a dose of 0.5 g/kg and was given intraperitoneally. Following transplantation, animals were kept on the heating pad and were afterwards returned to their cages.

2.7. Western blot

Western blot analysis was performed according to standard procedures. Protein concentration was measured using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were separated on 10% SDS-PAGE gel and transferred to PVDF (polyvinyl difluoride) membrane (Sigma-Aldrich). The membranes were blocked with 2% non-fat milk in PBS containing 0.05% Tween-20 (Sigma-Aldrich) for 45 min at room temperature and then incubated with primary antibodies at +4 °C overnight. Primary and secondary antibodies used for western blot analysis were listed in Table 1.)

2.8. Immunohistochemistry and immunocytochemistry

This method have been described previously [10] with modification of adding 10 mM sodium citrate which was used as antigen retrival for cells and tissues labelled with BrdU [11]. Primary and secondary antibodies used for immunohisto- and immunocytochemistry are listed in

Table 1
Primary and secondary antibodies used for Western blot analysis.

Primary antibodies			
Antibody	Host	Dilution	Producer
Actin	mouse	1:15000	Millipore (MAB1501)
Neuroligin 1	rabbit	1:1750	Millipore (AB15512)
PSD-95	rabbit	1:1500	Cell Sigaling (mAb #3409)
SynCAM 1	rabbit	1:2500	Millipore (ABT66)
Synapsin 1	rabbit	1:1500	Cell Signaling (#2312)
Secondary antibodies			
Antibody		Dilution	Producer
goat anti-mouse IgG H&L (HRP)		1:15000	Abcam (ab6789)
goat anti-rabbit IgG H&L (HRP)		1:3000	Abcam (ab6721)

Table 2
Primary and secondary antibodies used for immunohist- and immunocytochemistry.

Primary antibodies			
Antibody	Host	Dilution	Producer
BrdU	mouse	1:750	Cell Signaling (mAb #5292)
Cleaved Caspase-3	rabbit	1:400	Call Signaling (#9661)
GAP43	rabbit	1:500	Novus Biologicals (NB300-143)
GFAP	chicken	1:250	Abcam (ab4674)
Ki67	rabbit	1:300	Abcam (ab15580)
Laminin	rabbit	1:100	Sigma (L9393)
MAP2	chicken	1:1000	Abcam (ab5392)
Nestin	mouse	1:200	Millipore (MAB353)
Neuroligin 1	rabbit	1:200	Millipore (AB15512)
Neuroligin 1	mouse	1:400	Synaptic Systems (129 111)
PSD95	rabbit	1:250	Cell Sigaling (mAb #3409)
SMI-32	rabbit	1:500	Merck (NE1023)
SynCAM 1	rabbit	1:250	Millipore (ABT66)
Synapsin 1	rabbit	1:100	Cell Signaling (#2312)
Secondary antibodies			
Antibody		Dilution	Producer
Alexa Fluor 488 goat anti-chicken IgG		1:1000	Invitrogen (A11039)
Alexa Fluor 488 goat anti-mouse IgG		1:1000	Invitrogen (A11001)
Alexa Fluor 488 goat anti-rabbit IgG		1:1000	Invitrogen (A11034)
Alexa Fluor 546 goat anti-chicken IgG		1:1000	Invitrogen (A11040)
Alexa Fluor 546 goat anti-mouse IgG		1:1000	Invitrogen (A11003)
Alexa Fluor 546 goat anti-rabbit IgG		1:1000	Invitrogen (A11010)

Table 2.)

2.9. Cell counting

Number of positive cells was counted on photographs obtained by confocal microscope (LSM 510 Meta, Zeiss). Measurements were obtained using inbuild Zeiss software combined by macro adding from ImageJ. When graft regions were analyzed, average region size was 0.8 mm2 (0.6–1.2 mm2). Number of cells counted depended on the tested groups and it was in the range of 800 ± 143 to 3200 ± 465 cells. For quantification of cells *in vitro*, days 0, 1, 3, 5 and 7 were analyzed. Statistical analysis was performed by ANOVA with Bonferroni post hoc test (significance difference: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). The pooled mean of marker positive cells and pooled SD (Welch–Satterthwaite equation) was obtained by combining the results ($n = 5$) of three independent experiments. The results were rated independently by two evaluators and coincided 100%.

2.10. Statistical analysis

Data are reported as mean ± standard error of mean (error bars) of three independent experimental measurments, each performed in triplicate, for immunocytochemistry. An overall difference in samples data were analyzed by two-way analysis of variance (ANOVA) followed by Student T-test for pairwise comparison and Bonferroni post hoc test for multiple comparisons in order to test for significance between individual measurments and/or groups of surface markers. Significance level was set at $p = 0.05$ (significant differences between groups are marked by * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0001$).

3. Results

3.1. Standardized procedure of cell isolation and cultivation before transplantation yielded uniform cell populations

To test if our protocol yields reproducible and standardized neural stem cell population, their proliferation and differentiation potential was measured in three consecutive experiments. NSCs isolated from E14.5 fetal telencephalon proliferated *in vitro* as neurospheres in the presence of growth factors EGF and FGF. Their proliferation was followed by measuring the number of Ki67$^+$-cells. After removal of growth factors from the culture, the number of nestin$^+$-cells decreased within seven days from 99.3 ± 0.80% to 22.77 ± 22.61%, while the number of MAP2$^+$-neurons in the same period reached 73.4 ± 13.53%. Since GFAP$^+$-astrocytes require longer incubation, they were recognized as 4.8 ± 4.23% of the cells in the total population around the seventh day of cultivation (Fig. 1A). The procedure has been performed in three independent experiments. No significant effects in the total variability of differentiation have been observed (Fig. 1B). This revealed that our protocol yields cell populations which can be reliably compared in different experiments.

3.2. Expression of synaptic proteins gradually increased during in vitro and in vivo differentiation of NSCs

The expression of genes and the presence of synaptic proteins were observed in this study, using them as markers of early networking. Intercellular contacts were measured by expression of PSD-95, Nlgn1, SynCAM 1 and Syn1. The expression of synaptic proteins and their detection in cells were gradually increasing during seven days of NSCs differentiation, as shown by RT-PCR, Western blot and immunohistochemistry of all four synaptic proteins. Expression and presence of Nlgn1, SynCAM 1 and Syn1 increased on average five times in seven days of differentiation, while PSD-95 in the same period increased three times (Fig. 2).

Immunocytochemical analysis revealed a specific expression pattern for each marker. Nlgn1, SynCAM 1 and PSD-95 exhibited a strong perinuclear signal in the cytoplasm of undifferentiated nestin$^+$-cells. From the third day in *in vitro* conditions, all markers showed an intense punctate staining in differentiating MAP2$^+$-neurons. This probably represents preformed protein aggregates required for synapse formation. Nlgn1, SynCAM 1 and PSD-95 exhibited different expression pattern in dendrites. Nlgn1 exhibited the strongest signal expressed diffusely in the dendritic cytoplasm (not shown), while SynCAM 1 exhibited the weakest signal limited only to the initial part (Fig. 3). PSD-95 was mostly present in dendrites. Syn1, as a presynaptic protein, marking presence of more mature synapse had a specific and different expression pattern from other analyzed synaptic proteins. It exhibited a faint signal in nestin$^+$- cells, but also a punctate staining (present exclusively in growing axons) from the third day of a culture. With further cell growth and differentiation, Syn1$^+$- puncta could often be found adjacent to the postsynaptic somatic and dendritic membrane. After transplantation into the brain, the presence of the same synaptic proteins was found in transplanted cells (Fig. 3). This was in congruency with reports published by our group that expression pattern of different genes *in vitro* with great reproducibility mirrors expression of genes *in vivo* [10]. Interestingly, we did not observe any differences in expression of synaptogenic proteins in transplanted cells between the stroke affected and healthy control animals (not shown).

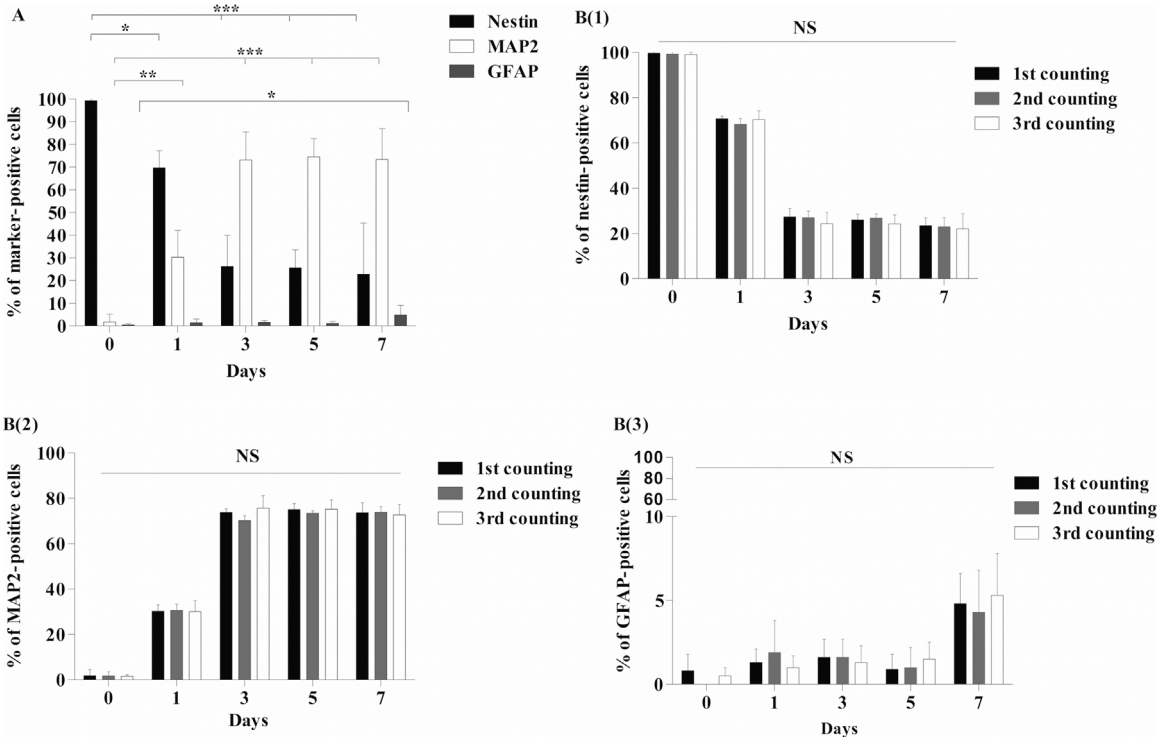

Fig. 1. Neural stem cells differentiation *in vitro*.
(A) Expression of nestin, MAP2 and GFAP in neural stem cells culture during seven days analyzed by immunocytochemistry. (B) Comparison of measurements of expression of markers in three independent experiments/counting. B(1) Expression of progenitor marker nestin was the highest in nondiferentiated NSCs (99.3 ± 0.80%), but decreased significantly during the next seven days of differentiation (22.77 ± 22.61%). B(2) expression of neuronal marker MAP2 increased significantly from 1.67 ± 3,55% to 73.4 ± 13.53% in a time-dependent manner. B(3) Increase in number of GFAP + -astrocytes was detected on the seventh day of neuronal differentiation with the value of 4.8 ± 4.23%. Number of positive cells for each marker (nestin, MAP2 and GFAP) was expressed as mean (%) ± SD of one quintuplicate experiment that was representative of three independent experiments. Statistical analysis revealed no significant differences in three independent experiments.

3.3. Behavior of transplanted neural stem cells depended on the host brain region

To test how different regions of the brain influence migration, survival and differentiation of grafted NSCs, the cells, marked by PKH26 and BrdU were transplanted into three different niches: the lateral ventricles, corpus callosum and the striatum. Neural stem cells transplanted into the corpus callosum migrated along the border of white and grey matter (Fig. 4A). In the healthy animals NSCs migrated a rather short distance not more than 1200 micrometers in both directions, while in the stroke-affected animals 85% of NSCs migrated 2500 micrometers towards the ischemic injury and populated the region of ischemia. In the same time, 15% of the cells have migrated toward the contralateral hemisphere (Fig. 4A). After reaching the ischemic region, NSCs have accumulated in both penumbra and the core of the stroke.

NSCs transplanted in the lateral ventricles of both healthy and stroke-affected animals survived in the cerebrospinal (CS) fluid for four weeks, which was the end point of our experiment. In that region, the cells were forming neurospheres like during their *in vitro* cultivation (Fig. 4B). Approximately 25% of NSCs started with spontaneous differentiation into $MAP2^+/NeuN^+/Tuj1^+$-neurons in the lateral ventricles, while majority of the cells remained in the nestin-positive multipotent state. In the healthy animals, NSCs remained exclusively in the ventricles, while in the stroke-affected animals 15% of cells migrated outside of the CS fluid into the surrounding brain parenchyma. This migration was noticeable on the stroke- affected side and the distance, measured from lateral ventricle, was on average not larger than 400 micrometers (Fig. 4B). In the following weeks, NSCs have differentiated into $MAP2^+/NeuN^+/Tuj1^+$-neurons. Number of cells, which have populated ischemia − affected region after transplantation in the lateral ventricle was 5 times lower when compared to animals in which cells have been transplanted in corpus callosum.

Neural stem cells transplanted in the striatum of healthy animals remained in the place of transplantation and undergo differentiation. In the stroke affected animals, a massive migration involving more than 75% of cells towards the ischemic region was observed. Since transplantation in this case was near the place of ischemia, migration was a rather short distance, on average 400 micrometers. The majority of cells accumulated in the region of penumbra or in the core of the ischemic lesion (fig. 4C).

3.4. Stroke improved the survival rates of transplanted neural stem cells in the striatum while it had no significant influence on their differentiation or on any of tested parameters of cells transplanted in other regions of brain

In this work we have shown that MCAO method combined with Doppler sensor leads to obtaining uniform brain ischemia – stroke affecting mostly the motor cortex and striatum of the experimental animal (Supplementary Fig. 1). In this study no statistically significant differences in survival and differentiation of transplanted NSCs between stroke affected and control animals into the lateral ventricle or corpus callosum were not observed. On the other hand, transplantation into the striatum revealed significant differences: four weeks after transplantation we have found 260 ± 19% more transplanted cells in the striatum affected by stroke than in the control animals. In order to check if the observed difference was related to hypothesized increased cell proliferation in the brain affected by ischemia, the number of $Ki67^+$-cells was counted. However, in both stroke affected and healthy animals, number of cells expressing Ki67 was 1.5 ± 0.7%. This suggested that proliferation might not be the reason for higher number of cells. Moreover, we measured presence of activated caspase 3 (Casp3). In the graft present in striatum of the brain affected by ischemia,

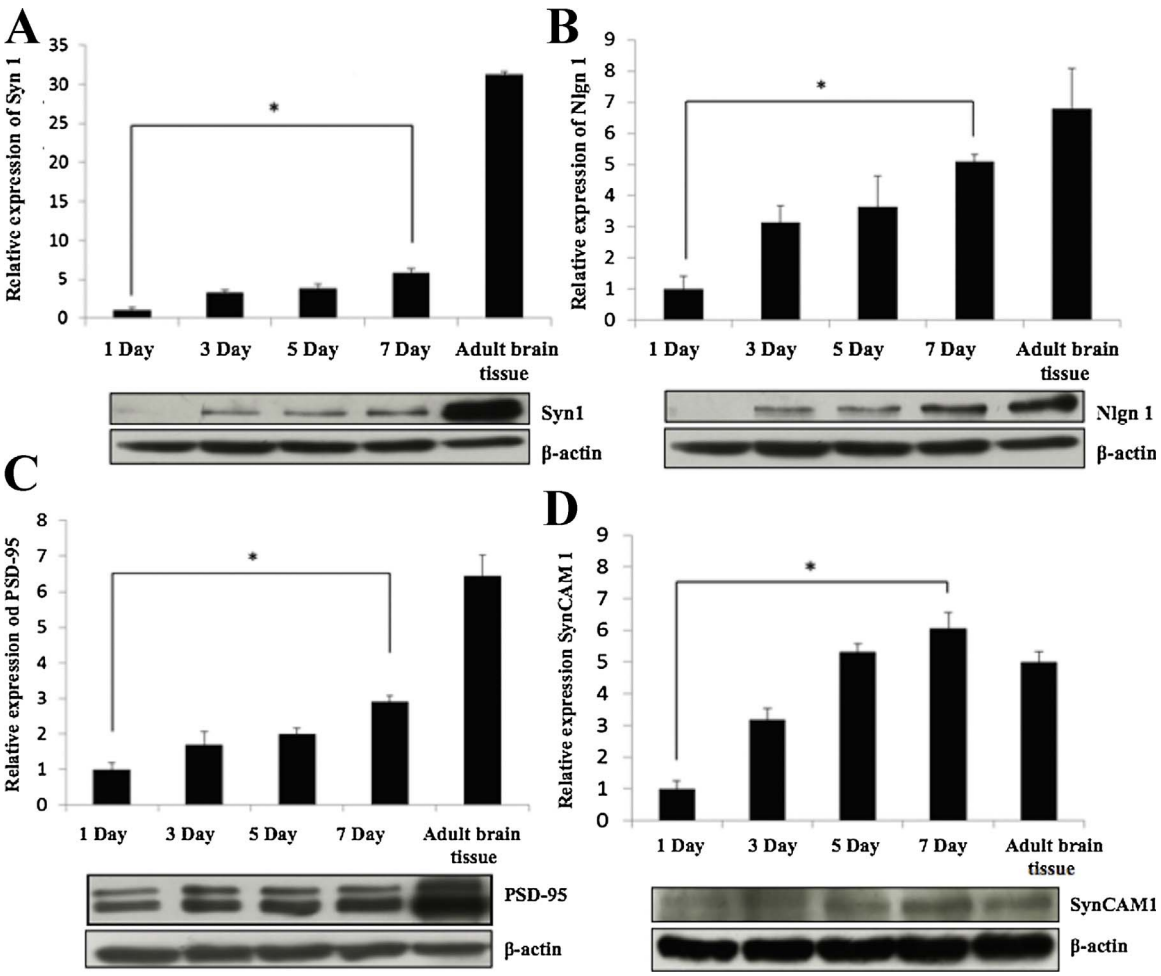

Fig. 2. Analysis of presence of synaptic markers in the culture of neural stem cells during differentation at days 1, 3, 5 and 7 measured by Western blot.
Statistically significant increase in the expression of all four synaptic markers, normalized to β-actin, was visible on the seventh day of differentiation when compared to the first day: (A) Syn 1, (B) Nlgn1, (C) PSD-95 and (D) SynCAM 1.

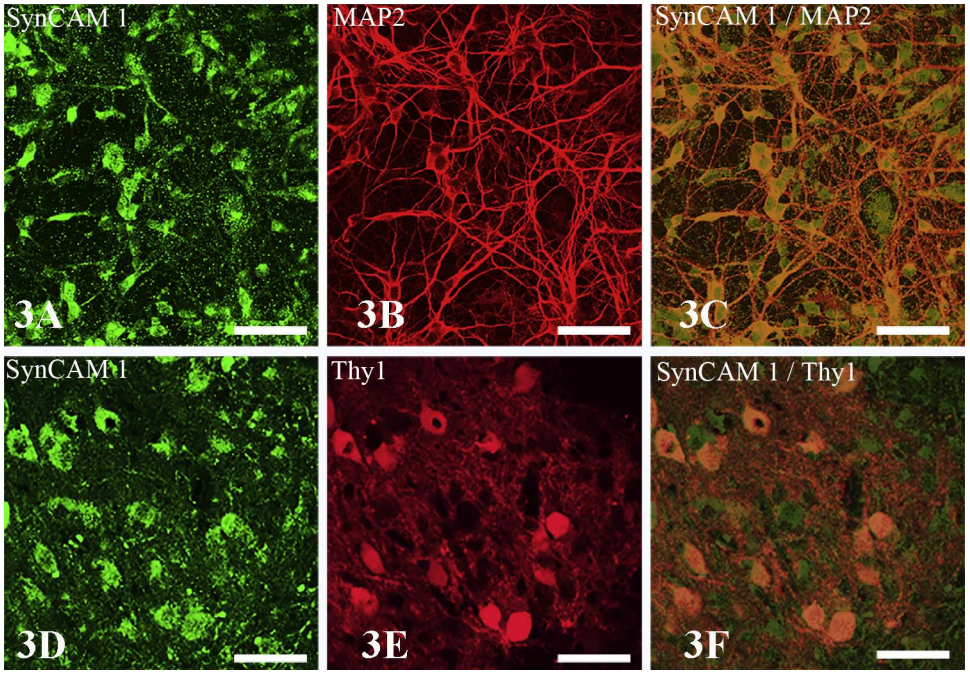

Fig. 3. Presence of SynCAM 1 in MAP2+ neurons (*in vitro*) and Thy1+ neurons (*in vivo*).
Double immunohistochemical visualization of SynCAM 1 (green) and either MAP2 (B and C) in *in vitro* or Thy1 (E and F) in *in vivo*. (A–C). SynCAM 1 expression detected in the cytoplasm of MAP2^{+}-neurons after 5 days of *in vitro* cultivation. (D–F) SynCAM 1 expression in transplanted Thy1 neural stem cells, detected after 4 weeks in the host brain tissue. Scale bars = 100 μm.

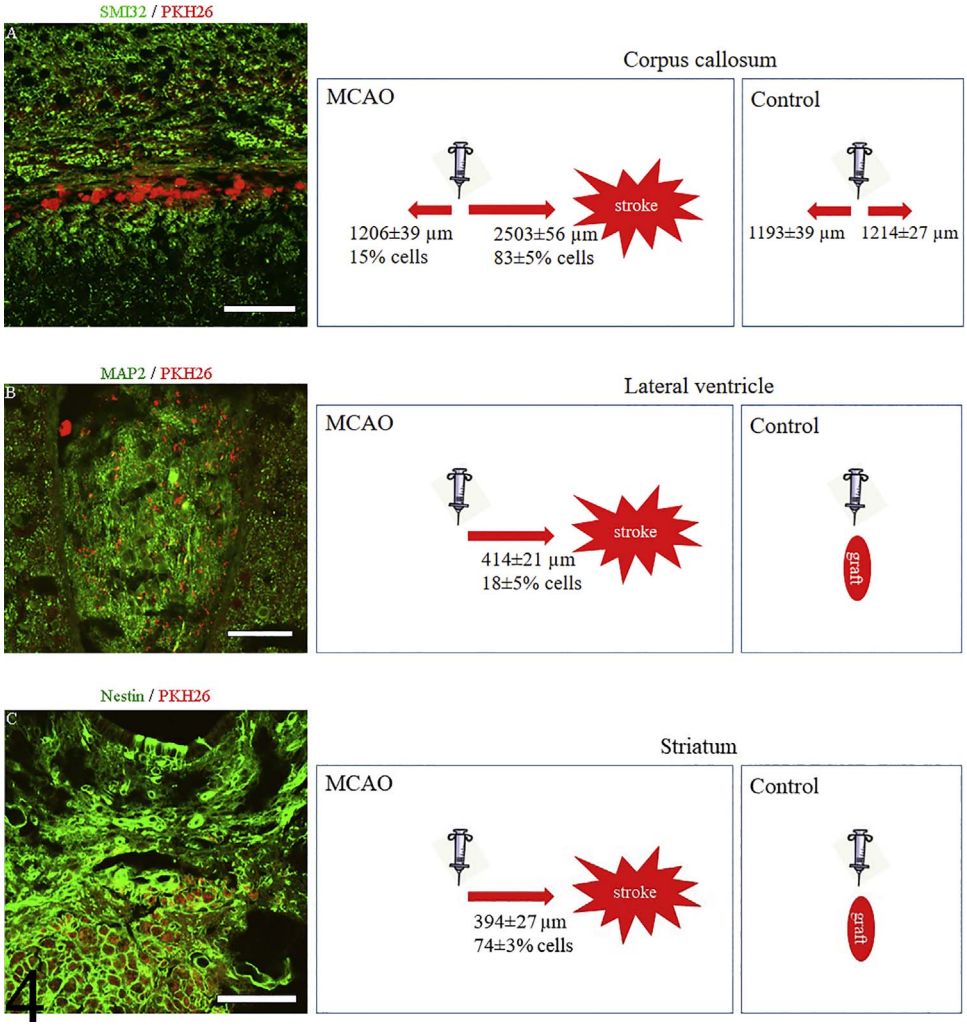

Fig. 4. Behaviour of NSC transplanted into the mouse brain.
Behaviour of PKH26 (red) labelled and transplanted NSC into the mouse brain affected by stroke and its healthy control. (A) Migration of transplanted NSC along the corpus callosum. 85% of the cells migrate towards the stroke and they travel on average 2500 µm. In the healthy control, they migrate equally in both directions and the average distance is 1200 µm. (B) 15% of the cells transplanted in the lateral ventricle of the stroke-affected animal migrate out of it and travel on average 400 µm towards the stroke. In the healthy animal cells remain in the ventricle. (C) 75% of the cells transplanted in the striatum of the stroke-affected animal migrate towards the ischemic region and the average distance is 400 µm. In the healthy animal cells remain in the region of injection. Scale bar 100 µm.

number of Casp3$^+$-cells after two weeks and even after four weeks (18.43 ± 2.6% and 19.56 ± 3.1%, respectively) was significantly lower when compared to healthy control after two weeks and after four weeks (28.59 ± 5.1% and 30.14 ± 2.4%, respectively) (Fig. 5). Therefore, it was suggested that larger size of the graft in the brain affected by ischemia after 2 and 4 weeks might be due to decreased apoptosis and not to increased cell proliferation.

Transplanted NSCs have differentiated into Thy1$^+$/MAP2$^+$/NeuN$^+$-neurons and GFAP$^+$-astrocytes in the injured brain as well as in the control. Again, there were no observed differences in differentiation of cells in the stroke-affected and healthy animals. The expression of synaptic proteins was detected in transplanted NSCs, however predominantly in differentiating neurons. Thy1$^+$-neurons were positive for Nlgn1, PSD-95 and SynCAM 1. SynCAM 1 showed a strong perinuclear signal, while PSD-95 and Nlgn1 had a stronger signal in the cytoplasm of the neurites. The expression of tested synaptic proteins in differentiated NSCs was present in cells transplanted in the injured as well as control tissue. Also the proportion of NSCs differentiating into either neurons or astrocytes did not differ between these two groups (Fig. 5).

3.5. Brain tissue affected by ischemia exhibited high level of remodeling, including increased expression of synaptic markers

Since we have observed that NSCs transplanted in the striatum survived better in the brain affected by stroke than in its healthy control, we searched for possible reasons that could be detected in the host tissue. Immunohistochemical expression of antibodies against laminin, GAP 43 and GFAP revealed intensive remodeling of the injured brain tissue which started two days after the initial insult and had lasted for more than four weeks (Fig. 6). Laminin signal was detected two days after ischemia and was getting stronger, thus suggesting increased angiogenesis especially in the penumbra, but also visible in the ischemic core. GAP 43$^+$-growing axons surrounded the ischemic core and bridged it on the periphery. GFAP$^+$-astrocytes showed an intensive reactive gliosis in the recovering brain. All observed processes (angiogenesis, activation of astrocytes and axonogenesis) could have contributed to increased graft survival discovered in the brain affected by ischemia.

Since our results showed that transplanted cells do undergo syanaptogenesis, it was also checked whether an increased synaptogenesis can be detected in the host tissue. Indeed, as opposed to the healthy brain, brain affected by ischemia exhibited a strong upregulation of all the synaptic proteins analyzed (Nlgn1, SynCAM 1, PSD-95, Syn1). Nlgn1$^+$-cells coming from the host were detected in migration towards the injured site. Moreover, Nlgn1$^+$-cells have been found in both ipsilateral and contralateral hippocampus of the brain affected by stroke (not shown). PSD-95$^+$-progenitors were much more exclusive and were discovered only in the small layer facing the ischemic core. All synaptic proteins were also strongly expressed in the outgrowing neurites in the penumbra (Fig. 6).

4. Discussion

In this work we showed that standardization of protocol leads to

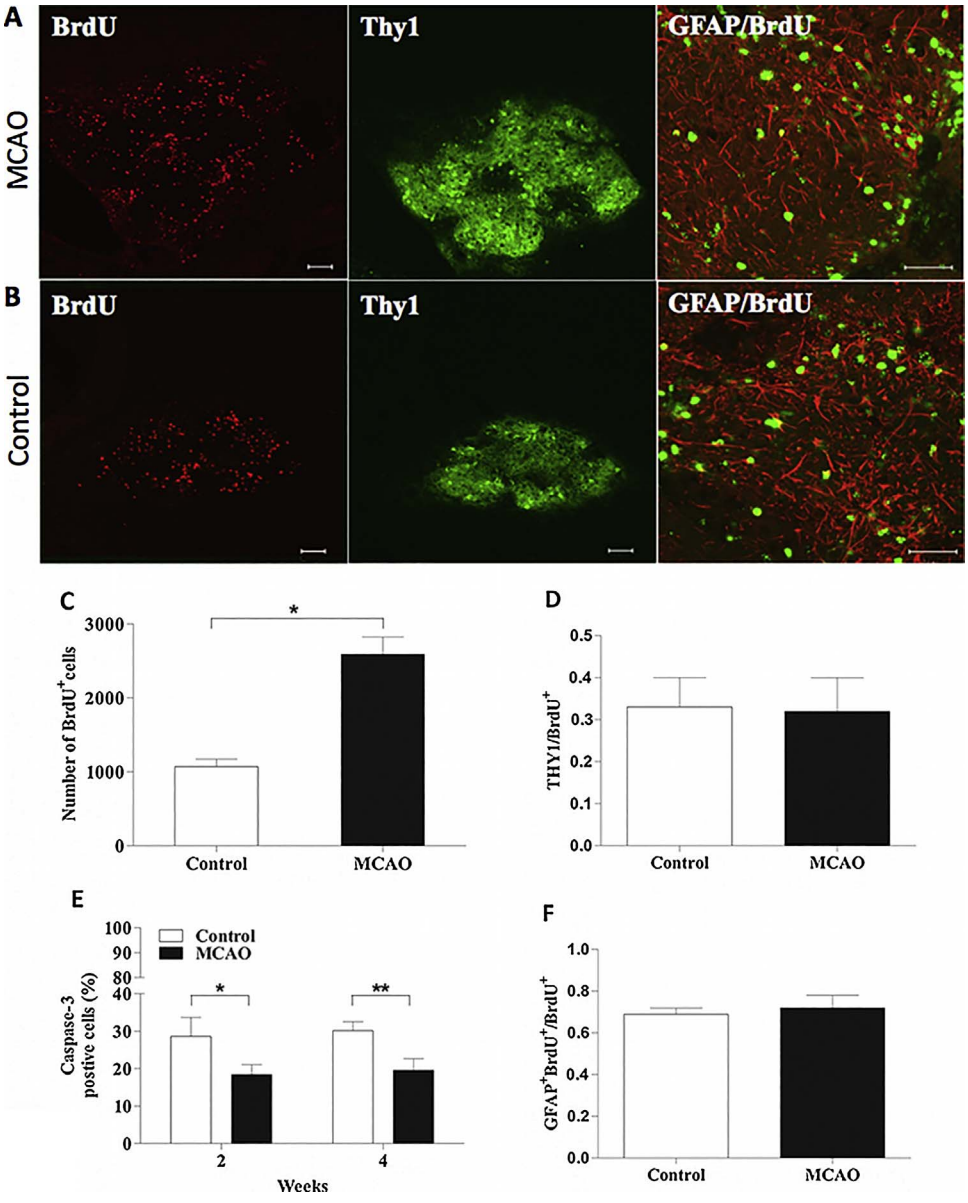

Fig. 5. Analyses of the fate of transplanted Thy1$^+$ neural stem cells into the stroke-affected brain and in its healthy control.
Comparison of the grafts in the brain affected by ischemia (A) reveals larger graft size after 4 weeks than in its wild type control (B). Quantification and comparison of graft sizes 4 weeks after transplantation (C): The survival of transplanted NSCs was more than two times higher in the injured tissue than in its healthy control (n = 4, *p < 0.05). Ratios of newly formed neurons (D) and astrocytes (F) compared to total number of cells in grafts in both stroke-affected and healthy control brains were the same. (n = 4, *p < 0.05). (E) Percentage of Casp3–positive cells 2 and 4 weeks after transplantation in the stroke-affected and healthy brain. Improved survival of transplanted NSCs observed in the stroke-affected brain was due to decreased cell death. The values of Casp3$^+$-positive cells are represented as the mean (%) ± SD (n = 3). Scale bar = 100 μm

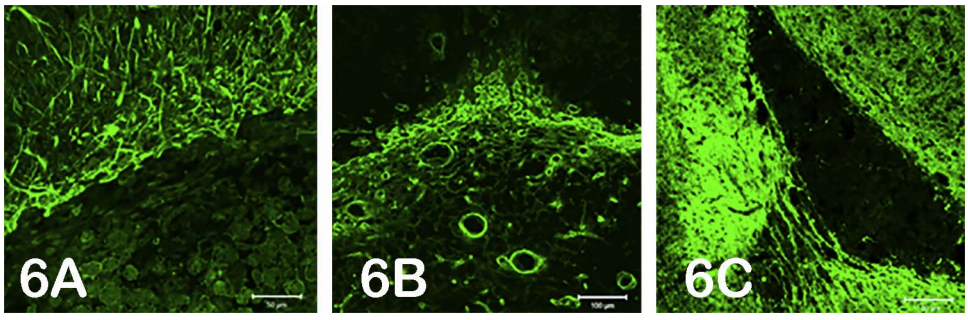

Fig. 6. Immunohistochemical presentation of reactive changes in postischemic brain tissue.
(A) Reactive gliosis at the border with ischemia-affected region detected by GFAP$^+$, (B) reactive angiogenesis in the penumbra marked by laminin$^+$-blood vessels and (C) ingrowth of GAP 43$^+$-axons from penumbra towards the iscehmic core. Scale bars = (A) 50 μm, (B and C) 100 μm.

obtaining a uniform population of NSCs with well-defined properties. *In vitro* testing of independent batches of cell samples revealed progressive differentiation towards neurons and glia in ratios which do not differ significantly in different experiments. This was an important finding because reproducible parameters of NSCs differentiation and survival are a prerequisite for analyzing behavior of these cells after transplantation in the brain of experimental animals. Our recent findings showed that the birth and differentiation of neurons after transplantation of NSCs in the host brain tissue follow the same pattern like the one observed in *in vitro* system and during embryonic development [10]. Taken altogether, the cell behavior, observed after transplantation in the brain, is mostly, if not exclusively, caused by features of the host tissue and not by differences among various stem cell samples.

Results obtained in the last two decades suggested that transplanted neural stem cells survive for some period in the host tissue where they

contribute to repair of damaged tissue [4,12]. This is achieved by at least three mechanisms: generation of new neurons [13,14], production of healing molecules [15] and promotion of endogenous neurogenesis [16]. This study has confirmed our previous findings that upon transplantation NSCs give rise to both neurons and glia. Although it is unclear to which extent it is required that transplanted stem cells survive and differentiate into region-specific cells, it is reasonable to measure the success of transplantation procedures based on survival of the transplanted cells. In this study, it was clearly shown that different regions of the brain influence differently behavior of transplanted NSCs. Therefore, NSCs transplanted in the lateral ventricles form neurospheres in the cerebrospinal fluid, which is similar to their *in vitro* behavior. We have been able to find some cells migrating towards ischemic region, but the number of migrating cells was very small. Majority of formed neurospheres remained attached to the ependyme and some of transplanted cells integrated into the ependymal epithelium. Interestingly, we have not observed major differences between stroke-affected mice and their healthy controls. Similar finding was reported by [17], who transplanted NSCs in the lateral ventricles of the healthy rat. Cells mostly remained attached to the wall and only some of them migrated towards the cortex. Park et al. [18] reported similar findings in healthy dogs, but they have also found cells in hippocampus and even in the spinal cord. It is possible that this difference is due to the relatively larger number of transplanted cells which leads to larger number of cells that migrate toward other regions of the brain. Similar publication reported events after transplantation of umbilical stem cells in the patient with global ischemia. Transplanted cells did not leave lateral ventricles even after 33 months of their monitoring [19]. This corresponds to our finding in the mouse with a massive stroke where cells mostly remain in the ventricles. Experiments in which cells have been transplanted in the lateral ventricles in the animal models of demyelination offered contradictory results: while [20] reported that human glial precursors cells do not leave injection point, [21] reported massive migration out of ventricles.

Transplantation of NSCs into the corpus callosum of the stroke affected brain results in their migration towards the injured region. This is in a congruency with reports which describe how cells migrate using white matter tracts [22]. This work has shown that the cells migrate toward the ischemic area and in lower extent towards the uninjured contralateral hemisphere. It would be interesting to perform post-transplantation analyses of these cells and check if there are some differences in expression of surface markers which could be linked to migration in the specific direction. It is possible that some cells do not detect signals present in the tissue affected by stroke, so their migration in ipsilateral or contralateral direction is occuring with the same probability. One could discuss why cells which are not attracted by ischemia do migrate at all. The reason for that might be that cells search for a differentiation niche. On the other hand, transplantation in the striatum leads to their survival in a large number at the place of transplantation. Afterwards, they migrate towards penumbra and in ischemic core and, after reaching their target, majority of them undergo differentiation [24],6,23]. Recently, de la Rosa-Prieto and colleagues reported that brain ischemia significantly influenced migration and axonal projections of human skin derived iPSCs. Transplanted cells migrated toward ischemic lesion and no difference in survival, proliferation or differentiation between cells transplanted in stroke affected and healthy tissue have been observed [24].

One of the most important results of this study was observation that transplanted NSCs survive in larger number in the niche affected by inflammation than in its wild type control. By counting number of $Ki67^+$ and $Casp3^+$-cells, it was suggested that larger number of cells found in stroke-affected brain compared to its control was due to decreased apoptosis of NSCs and not to their increased proliferation. To reliably follow transplanted cells, they have been marked by PKH26 and BrdU, which allow precise tracing of cells up to 6 weeks after transplantation. Multiplication of cells slowly dilutes the signal, but for the needed period of 4 weeks, its presence was still clearly visible. Interestingly, stroke-affected brain did not influence differentiation of NSCs, so the observed ratio of neurons and glia was the same in the stroke affected brains and in their wild type controls. The same was observed when comparing ratio of presence of synaptogenic markers, which suggests that stroke influences survival of cells, but not their differentiation pattern. Although it has been shown that the core of ischemia or a late stage neuroinflammatory disease is hostile for cell transplants, there are evidences from both preclinical and clinical trials suggesting that stem cells are much more attracted to disease-affected nervous tissue than to its healthy control. Equally, if cells find their niche, they survive longer in regions affected by reversible inflammation than in completely healthy tissue. In this work we suggest that at least one part of this improved survival is linked to decreased apoptosis. Recently, new data have started to reveal new roles of Casp3. While its activity is primarily linked to programmed cell death, changes in its levels are also linked to re-organization of the brain tissue. Our group has reported that immediately after stroke, a high expression and presence of activated Casp3 can be detected and this is linked to colocalization with GAP 43 [25]. This process is linked not only to cell removal after stroke, but also to reorganization at the synapse level. It has been reported that Casp3 is involved in differentiation of cells [26,27] and elimination of synapses, thus its downregulation might be the mechanism involved in adaptation on ischemia needed for cell survival. Indeed, there is a growing list of evidences that decreased activity of Casp3 is an important mechanism involved in protection against ischemia [28,29]. Further analyses will be needed to understand molecular pathways behind observed increase of expression of Casp3 in the brain affected by ischemia and decreased expression of Casp3 in the transplanted neural stem cells.

Our *in vitro* experiments revealed that NSCs express markers of synaptogenesis and their pattern of expression increases gradually over time, following their differentiation. Here we showed that Nlgn1 is already expressed after 24 h of cell cultivation. Acting as a postsynaptic cell adhesion protein which interact with neurexin, neuroligin is a potent inducer of formation of synapse. Its early presence during NSCs differentiation in *in vitro* conditions thus suggest that synaptogenesis might be occuring symultaneously with their transformation into neurons. Interstingly, our results showed that Nlgn1 is expressed very early on GFAP + -cells. This might be the earliest sign of the synaptic triad. Our study has detected PSD-95 during differntiation of NSCs, thus confirming the well known fact that PSD-95 comes in the contact with Nlgn1, which is one of the key events for synapse formation [30,31,32]. While Nlgn1 is required for the formation of excitatory synapses [33] (Kwon et al., 2012), it seems that coordinated activity of Nlgn1 and PSD-95 leads to the formation of dendritic spines [33,34]. Our finding is similar to the one reported by [35] who have recognized PSD-95 as one of the major markers of early synaptogenic activity of transplanted NSCs.

Importantly, there was no difference in the expression of synaptogenic proteins in transplanted NSCs between stroke-affected brain and its healthy control. This suggests that expression of synaptogenic proteins is process occurring independently of the host niche. Since we have shown that NSCs survive better in an ischemic niche, we have tested several markers of tissue reorganization which might promote cell survival. We found increased expression of laminin, GFAP and GAP 43. Increased expression of laminin is a natural reaction of the brain linked to increased neuronal regeneration. The role of laminin in mobilization of neuroblasts and formation of new neurons has been showed in *in vitro* experiments [36]. Increased expression of GAP 43 is possibly linked to at least two processes: GAP 43 has a central role in sprouting of new axons after injury, but also regulates the organization of presynaptic terminal and neurotransmitter release. The role of GAP 43 and its link to Casp3 has been discussed in details in the publication of our group [25] (Gorup et al., 2015).

Conflict of interest

The authors have no competing financial interests in relation to the work presented.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neulet.2017.12.040.

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