ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2013, 73: 116–129

Differentiation of glia-committed NG2 cells: The role of factors released from hippocampus and spinal cord

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The NG2-positive cells are the oligodendrocyte precursors, which, when terminally differentiated, are capable of myelinating the central nervous system. There is however an ever-growing list of evidences that NG2 cells actually possess an intrinsic neurogenic potential and they are capable of neuronal differentiation in response to environmental stimuli. To address the question, we have established a model of an indirect co-culture system of the freshly isolated rat neonatal NG2 cells and organotypic slices derived from two distinct CNS regions (hippocampus and spinal cord) to mimic the nervous tissue microenviroment. The cell differentiation in microenvironment of OGD-injured hippocampal slices has been studied as well. The molecular analysis of selected trophic factors has been performed to determine the patterns of their expression. Indeed, the comparison of the cell commitment and development in various microenvironments has pointed to significant dissimilarities. First of all, the medium being continuously conditioned by the hippocampal slices efficiently promoted neurogenesis. The effect has been significantly abolished in co-cultures with the injured tissue. The less pronounced susceptibility to adopting neuronal phenotype and the considerable slowdown of oligodendroglial development was observed in the co-cultures with the spinal cord slices. The role of BDNF in oligodendroglial progenitor commitment and development has been investigated proving that it is one of the key players in the examined processes. The specificity of the instructive clues cocktail might module the fate choice of mobilized endogenous or transplanted cells, which should be taken into consideration while planning neurorepair strategies.

Key words: NG2 progenitors, OPCs, glia, neurogenesis, trophic factors, local microenvironment, co-culture, organotypic slices

IINTRODUCTION

The NG2 proteoglycan-expressing cells (Stallcup 2002) are the oligodendrocyte precursors, which terminally differentiated, are capable of myelinating the central nervous system (CNS). They constitute up to 5% of total cells in mammalian CNS (Dawson et al. 2000) and have been shown to gather their migratory and proliferative potential throughout the lifespan (Nait-Oumesmar et al. 1999, Chari and Blakemore 2002, Tamura et al. 2007, Magnus et al. 2008). Actually, they represent the main, abundant (~70%) population of dividing cells in both young and adult CNS (Horner et al. 2002, Dawson et al. 2003). Recently, Ge and coworkers (2009) have reported their observation of the proliferative capacity of the NG2 cells characterized by multiple, branched processes. Those desirable, uncommon features point to these oligodendroglial precursors as the valuable candidates in neurorepair strategies.

Furthermore, numerous *in vivo* evidences show that these cycling cells are mobilized and expand in response to various chemical, immunological or traumatic injuries (Keirstead et al. 1998, Reynolds et al. 2002, Watanabe et al. 2002, Liu and Shubayev 2011, Lee et al. 2013). They have been shown to repopulate the damaged area replenishing a cellular deficit and eventually to develop into myelinating oligodendrocytes with

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Received 15 January 2013, accepted 11 March 2013

regenerative potential efficiently contributing to CNS remyelination (e.g. Lytle and Wrathall 2007, Kerr et al. 2010, Zawadzka et al. 2010). Even if the failure in oligogliogenesis has been noted, the local improvement has been often observed owning probably to the trophic support provided by the precursors. A spectrum of active compounds (complements, cytokines, chemokines, trophic factors) expressed by neural stem cells/progenitors could significantly modify local microenvironment making it more permissive to restorative processes (Ubhi et al. 2010, Cao et al. 2010).

The oligodendrocytes are known to be a source of trophic factors (e.g. IGF-1, GDNF, BDNF, NGF, NTF-3) that could eventually influence the adjacent cells (Wilkins et al. 2001, Dai et al. 2003, Zhang et al. 2006). Presumably, their precursors also express numerous factors which vary during cell development providing temporal instructive signals.

Over the past decade, it has therefore been debated whether the NG2 cells are indeed lineage restricted precursors. Until recently they have been regarded as the gliogenic (astro- and oligodendroglial) progenitors. There is however an ever-growing list of evidences that NG2 cells actually possess an intrinsic neurogenic potential and they are capable of neuronal differentiation in response to environmental stimuli (Belachew et al. 2003, Gaughwin et al. 2006, Sypecka et al. 2009).

In our previous study we have shown that freshly isolated, neither propagated nor further stimulated NG2 cells seeded onto hippocampal slices, readily integrated into tissue and differentiated both into neurons and glia (Sypecka et al. 2009). Moreover, despite a 7 day long co-culture, a certain population of NG2 cells retained nestin expression and proliferative capacity. Those findings contribute to the evidences detailed above suggesting that the NG2 cells are multipotential neural stem cells rather than lineage-restricted progenitors.

Assuming, that glia and neurons are functionally interdependent, the cell to cell stimulation is desirable and highly probable in restorative cascades following various CNS insults. It remains however to be substantiated that NG2 cells are capable of differentiating in response to soluble microenvironmental cues that are thought to play a role in mobilization of the endogenous NG2 precursors. To address this question, we have established a model of an indirect co-culture system (i.e. contact only by the culture medium) of organotypic slices to mimic the tissue microenviroment and the freshly isolated rat neonatal NG2 cells. Additionally, two distinct CNS regions (hippocampus and spinal cord) have been selected for the study with the aim of examining the potential response of NG2 cell to a different composition of environmental stimuli.

METHODS

NG2 progenitor cell isolation

Brain cerebral hemispheres from neonatal Wistar rats, bred in the Animal Care Facility of the Medical Research Institute (Warsaw, Poland), were used to prepare mixed glial primary cell cultures. All procedures were approved by IV Local Ethics Committee on Animal Care and Use (Ministry of Science and Higher Education). Briefly, isolated tissue was dispersed mechanically initially with Pasteur pipette and than with 22-μm needle. The resulting cell suspension was filtered using 41 μm Hydrophilic Nylon Net Filter, (Millipore, Bedford, MA), spun down (1500×g, 10 min) and seeded into 75-cm^2 culture flasks coated with 0.1 mg/ml poly-L-lysine. The culture medium was changed every second days. After 10–12 days in Dulbecco's (Gibco) medium (high glucose) with 10% fetal bovine serum and supplemented with penicillin-streptomycin, oligodendrocyte precursor cells were isolated according to the modified procedure of McCarthy and de Vellis (1980), based on different adhesion properties of particular neural cell types. Cell cultures were rinsed with complete medium and shaken first for 1 h on an orbital shaker (180 rpm) at 37°C to remove the microglial fraction, then (after medium replacement) for additional 15–18 h, with the aim of gently detaching the oligodendrocytes. .Progenitors obtained by this sequential dislodging method were spun down (1500×g, 10 min), mechanically dispersed with the 22-μm needle in F12/DMEM medium supplemented with Insulin-Transferrin-Selenium-A Solution (Invitrogen) and then filtered through 41-μm Millipore membranes. The population of single NG2 progenitors, suspended in 10 ml of culture medium, was placed in a 75-ml Falcon flask for 4 h in order to eliminate potentially contaminating cells (glia and neurons from the primary culture). The supernatant was gently collected and, finally, the purified $NG2^+$ population was seeded at $2\times10^5/cm^2$ density on poly-L-lysine-coated cover slips placed in 6-well plates (NUNC, Naperville, IL). In control experiments aimed at verification if the examined population is not getting contaminated with slice-derived cells

during culturing, the glial progenitors were labeled (30 min at 37°C) with cell tracker CMFDA (Invitrogen) prior to seeding.

Organotypic hippocampal culture (OHC)

Hippocampi were isolated from 7–8 day-old Wistar rats according to the method of Stoppini and coworkers (1991) and used for the organotypic slice preparation. After brief anesthesia with pentobarbital (Vetbutal, Sigma, St. Louis, MO, USA), ice-cooled pups were decapitated with scissors, then brains were quickly removed and placed into ice-cold HBSS (Gibco). Hippocampi were cut into 400-μm slices using a McIlwain tissue chopper and placed onto Millicell-CM (Millipore) membranes, four slices per insert. Millicell-CM membranes in 6-well plates were pre-equilibrated with 1 ml of culture medium (pH 7.2; DMEM 50%, HEPES,HBSS 25%, horse serum 25% (Gibco), 2 mmol/l L glutamine, 5 mg/ml glucose, 1% amphotericin B and 0.4% penicillin-streptomycin). The serum concentration in culture medium was gradually lowered. From 7th DIV slices were cultured in the serum-free conditions in DMEM/F12 containing HEPES, HBSS 25%, 2 mmol/l l-glutamine, 5 mg/ml glucose, 1% amphotericin B, and 0.4% penicillin-streptomycin, N2 (1:10; Gibco) and B27 (1:100; Gibco) supplements. Cultures were maintained in a moist atmosphere (95%) of air with 5% CO_2, at 36°C for 7–14 days and the medium was changed every second day.

Organotypic spinal cord culture (OSC)

The spinal cords were extracted from the same animals used for the hippocampi isolation. After dissection, 10 mm longitudinal sagittal slices of spinal cord were cut using a tissue chopper (McIlwain) at a thickness of 350 μm and transferred onto a permeable Millicell-CM (4 slices per membrane) and cultured according to the protocol used for OHC.

Oxygen-glucose deprivation injury to OHC

Oxygen glucose deprivation (OGD) was performed on 7 DIV OHC. The slices were transferred to an anaerobic chamber and placed in Ringer solution containing 10 mM mannitol, saturated with a mixture of 95%N_2/5%CO_2 for 40 min. Cell death in the organotypic cultures was evaluated 24, 48, 72 h, and 7 days after OGD to estimate the degree of tissue damage.

Evaluation of cell death

Quantification of cell death was performed as previously described (Pozzo Miller et al. 1994, Cavaliere et al. 2005). The fluorescent cell death marker Propidium iodide (PI) was added to the medium 24 h prior to the experiments and throughout the recovery period. Fluorescent images were acquired using a confocal microscope (Carl Zeiss LSM 510), equipped with ZEN 2008 software. The PI-based examination was performed prior to OGD for selecting the undamaged slices, while after OGD the slices with a similar, high degree of tissue damage were chosen for co-culture experiments.

Damage was detected only in the cornu ammonis (CA) area for evaluation of the neuronal damage. Number of dead cells was calculated for each standardized CA region as follows: % of dead cells = (experimental fluorescent intensity (FI) − background FI) / (maximal FI − background FI) × 100.

Co-culturing of NG2 cells with organotypic slices (OHC/OSC)

The freshly isolated and purified NG2 population was seeded at $2\times10^5/cm^2$ density on poly-L-lysine-coated cover slips placed in 6-well plates (NUNC, Naperville, IL) and left to adhere for approximately 1 h. Then the Millicell-CM membranes containing either hippocampal intact slices (OHC), OGD-subjected hippocampal slices (OHC/OGD) or spinal cord slices (OSC) were transferred to the plates with NG2 progenitors and closely co-cultured for the 5 following days in serum-free DMEM supplemented with antibiotic solution AAS (Gibco). The culture medium was changed every second day. On the 5 DIV, the cell cultures were treated for 20 min with 4% PFA for immunocytochemical studies or gently tripsynized (Sigma) for 5 min and stored for RNA isolation. Organotypic slices (OHC, OHC/OGD, OSC) were also collected for RNA extraction and either used immediately or kept in RNAlater Solution (Applied Biosystems) in 4°C.

Endogenous BDNF neutralization

In blocking experiments, the anti-BDNF antibody (Millipore) was added in an excessive amount (10 μg/ml) to the media at the time of the co-culture set-up and at every 24 h. At 7 DIV, the cultured cells were processed for immunocytochemistry.

Cell treatment with the exogenous BDNF

For analysis of the potential effects of exogenous BDNF on the NG2 cell differentiation, the BDNF (Sigma) at concentration of 20 ng/ml was added to the culture medium at the time of plating and after 48 hours.

Estimation of BDNF concentration in organotypic slices and co-culture media

Culture media were collected and then concentrated by a factor of 30 X and desalted by centrifugation at 3 000×g (30 min) using Spin-X UF concentrator (Corning) filter (molecular weight cutoff 10 kD). Organotypic (hippocampal and spinal cord) slices were transferred to CelLytic™ MT cell lysis buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma) and gently homogenized. Protein concentration was determined by the modified Lowry method (Lowry et al. 1951). BDNF concentrations were evaluated by ChemiKine Brain Derived Neurotrophic Factor, Sandwich ELISA (Millipore), according to the supplier's instructions. After the reaction had been stopped, the plate was read at 450 nm using a spectrophotometric plate reader Fluorostar Omega (BMG LabTech).

Immunofluorescent staining

Blocking solution containing 10% normal goat serum in PBS, was applied for 1 h at 25°C (RT). For cytoskeletal markers, cells were permeabilized for 20 min in PBS containing 0.01% Triton and 5% normal goat serum in PBS. Immunostaining with primary antibodies was carried out by overnight incubation at 4°C. After rinsing the cells with PBS, they were incubated for 1 h at room temperature with an appropriate secondary antibody conjugated to either Alexa-488 or Alexa-546 (1: 1000, Molecular Probes). Controls for specific immunostaining were stained omitting either the primary or the secondary antibody. Markers for different stages of oligodendrocyte development were used: rabbit polyclonal anti-NG2 (1:200, Chemicon, Temecula, CA) and monoclonal anti-mouse against O4 (1:200, Sigma), CNP-ase (Sigma, 1: 500), GalC (1:200, Chemicon) and MBP (1:100, Sigma). To identify differentiating neurons, the following monoclonal anti-mouse markers were applied: anti-NF200 (1:200, Sigma), TUJ1 (β-tubulin III) (1:500, Sigma) and anti-MAP2 (1:500, Pharmingen, San Diego, CA). Additionally, Ki67 – the indicator of proliferating cells (1:100, Novocastra, Newcastle Upon Tyne, UK) and nestin – a marker of neural stem cells (1:250, R&D Systems) were used. Precursor characteristic of the cells was verified by applying rabbit polyclonal anti-PDGFRα (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal antibody anti-A2B5 (1:1000, Chemicon). Cell nuclei were visualized by incubation with 5 μm Hoechst 33258 (Sigma). Labeled oligodendroglial cultures were examined using an Axiovert 25 fluorescence microscope. Images were captured by the Videotronic CCD-4230 camera (Carl Zeiss, Jena, Germany) and processed by the Axiovision (Carl Zeiss) image analysis system. Confocal microscope along with LSM 510 software package version 3.2 (Zeiss) was used to analyze the immunohistochemical images (labeled OHC and OSC). The argon laser (488 nm) and helium-neon laser (543 nm) enabled visualization of Alexa-stained cells.

Cell counting

Each experiment was repeated four times and 3 representative slides with immunolabeled cells have been selected for determination of distribution of both cell type and maturation stage specific markers. The cells were manually counted in four randomly selected fields within each of the three slides. The number of the marker-positive cells was calculated against the total number of Hoechst-stained cell nuclei within the examined field (considered as 100%). Since the markers attributed to the sequential stages of the either oligodendroglial or neuronal differentiation were often overlapping, the quantities of populations determined by the applied markers were not the additive values.

Reverse transcription and quantitative real-time RT-PCR analysis

Total RNA was extracted by Trizol reagent (Invitrogen) and 1 μg of each sample was used for reverse transcription reaction High Capacity RNA-to-cDNA Kit (Applied Biosystems), according to the manufacturer's instructions. Real-time PCR analyses were performed in ABI Prism 7500 Sequence Detection System using 50 ng of cDNA, designed specific primers (listed in Table I) and SYBR Green PCR Master Mix (Applied Biosystems).

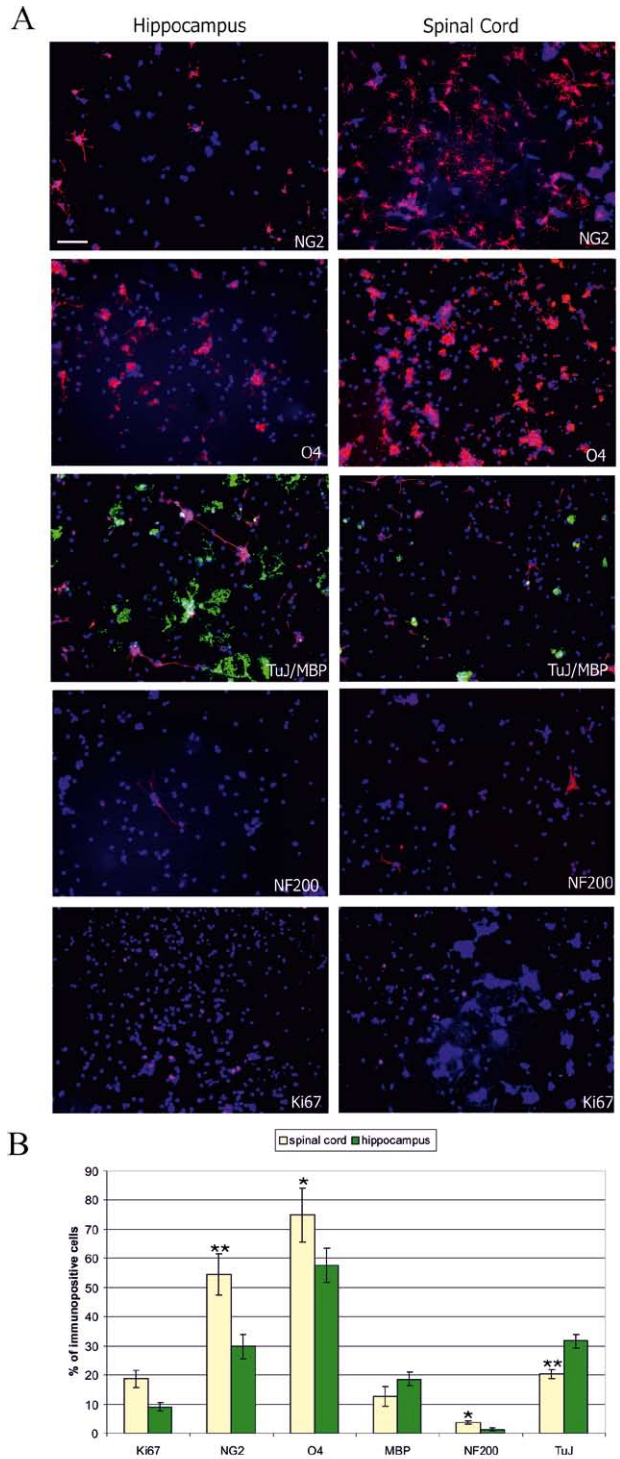

Fig. 1. Differentiation of NG2 precursors in co-culture with the organotypic slices of either hippocampus or spinal cord during 5 DIV. (A) Immunocytochemical analysis of cells differentiated in co-culture with hippocampal (left panel) and spinal cord (right panel) slices. Cell nuclei are visualized by Hoechst 33258 immunostaining (blue). The photos of double-staining show the expressions of neuronal marker TUJ (red) and oligodendroglial marker MBP (green) expressions, respectively. Scale bar is 50 µm. (B) Comparative analysis of major neural markers and Ki67 expression in 5 DIV cell co-cultures (with hippocampal slices vs. spinal cord slices). All values on the graphs were expressed as mean ± SEM. Differences were considered significant if: **P*<0.05; ***P*<0.01

Table I

A list of designed primers used in reverse transcription and quantitative real-time RT-PCR analysis

Gene	Accession No.	Forward primer sequence	Reverse primer sequence	Product length (bp)
Actb	NM_031144	TCTTGCAGCTCCTCCGTCGC	ACGATGGAGGGGAAGACGGC	150
BDNF	NM_012513	CGGCTGGTGCAGGAAAGCAA	TCAGGTCACACCTGGGGCTG	136
CNTF	NM_013166	CACCGCCGGGACCTCTGTAG	GCTTGCCACTGGTACACCATCC	147
GDNF	NM_019139.1	AAGGTCGCAGAGGCCAGAGG	TCTCGGCCGCTTCACAGGAA	144
IGF-1	NM_178866.4	CAGCATTCGGAGGGCACCAC	CATGTCAGTGTGGCGCTGGG	145

The NGF level was determined by application of RT^2 Profiler™ PCR Array for rat neurotrophins (SABioscience) following the manufacturer's instructions. Reaction parameters were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The dissociation curve was plotted to determine the specificity of amplification. The products were separated against Low Range DNA Ladder (Fermentas) in 1.5% agarose gel to verify their size. The samples were collected in at least four independent experiments. Each sample was tested in triplicate during three analyses. The fluorescent signals from specific transcripts were normalized against that of the β-actin gene and threshold cycle values (ΔCt) were quantified as fold changes by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

The data gathered in experiments comparing the three experimental groups and control were subjected to statistical analysis with one-way analysis of variance (ANOVA) followed by *post-hoc* Fisher's test. A non-paired student *t*-test (GraphPad PRISM 5.0) was applied in order to analyze differences between a given group and the control. All values on the graphs were expressed as mean ± SEM. Differences were considered significant if: $*P<0.05$; $**P<0.01$.

RESULTS

Neurogenic effect of microenvironments on progenitor fate-choice

The aim of the present work was to investigate the influence of distinct neural microenvironment on the naive NG2 precursor development. To avoid any additional stimuli, the cells were isolated (~97–98% viability at the end of the procedure), shortly purified and immediately used for the co-culture experiments. As in our previous study (Sypecka et al. 2009), the simple protocol aimed at minimal manipulation of the cells before their seeding, allowed us to obtain a homogenous population (98 ± 3.31%) of NG2$^+$ glia-committed (95 ± 2.78% PDGFRα$^+$; 96 ± 5.25% A2B5$^+$; 79.48 ± 2.78% CNP$^+$) progenitor cells, with a diameter of about 9–11 μm. After 5 DIV in co-cultures, the cell differentiation was assessed by immunocytochemical and molecular analyses. In control experiments, the freshly isolated progenitors were labeled with CMFDA prior to setting up the co-cultures with the aim of verifying whether the developing NG2$^+$ population was not contaminated with the slice-derived cells. The cells cultured without organotypic slices differentiated within 7 DIV into monoculture of mature oligodendrocytes, lacking any neuronal marker expression.

The comparison of the cell commitment and development in the microenvironments provided by tissue derived from distinct neural regions (Fig. 1A,B) showed significant dissimilarities. First of all, the medium continuously conditioned by the hippocampal slices efficiently promoted neurogenesis, resulting in a sizeable fraction of TUJ-positive cells (31.6 ± 6.7%) accompanied by a marginal fraction of NF200$^+$ (1.36 ± 0.6%) early neurons. The medium conditioned by the spinal cord slices had also neurogenic effect, yet to much a lesser extent (20.3 ± 4.1% of TUJ-positive cells) when compared to that observed in co-cultures with hippocampal slices. It is worth noting, that the number of neurons at the primary stage (NF200$^+$) of maturation, although still marginal, was twofold higher though (3.75 ± 1.4%) in this microenvironment (Fig. 1B).

Influence of both types of co-cultures on oligodendroglial development of NG2 cells

The examination of oligodendroglial markers attributed to sequential developmental stages also revealed substantial distinctions. First of all, no significant decrease in the total cell number was observed in co-cultures with the OGD-subjected slices in comparison with both control oligodendrocyte monoculture, as well as with control co-cultures. What is strikingly noticeable is that the fraction of the multibranched NG2 cells was relatively high (55 ± 14.2%) in the microenvironment provided by spinal cord slices in comparison with that created by hippocampal slices (29.8 ± 9.8%). The marker of immature oligodendrocytes O4 was also significantly elevated, while MBP

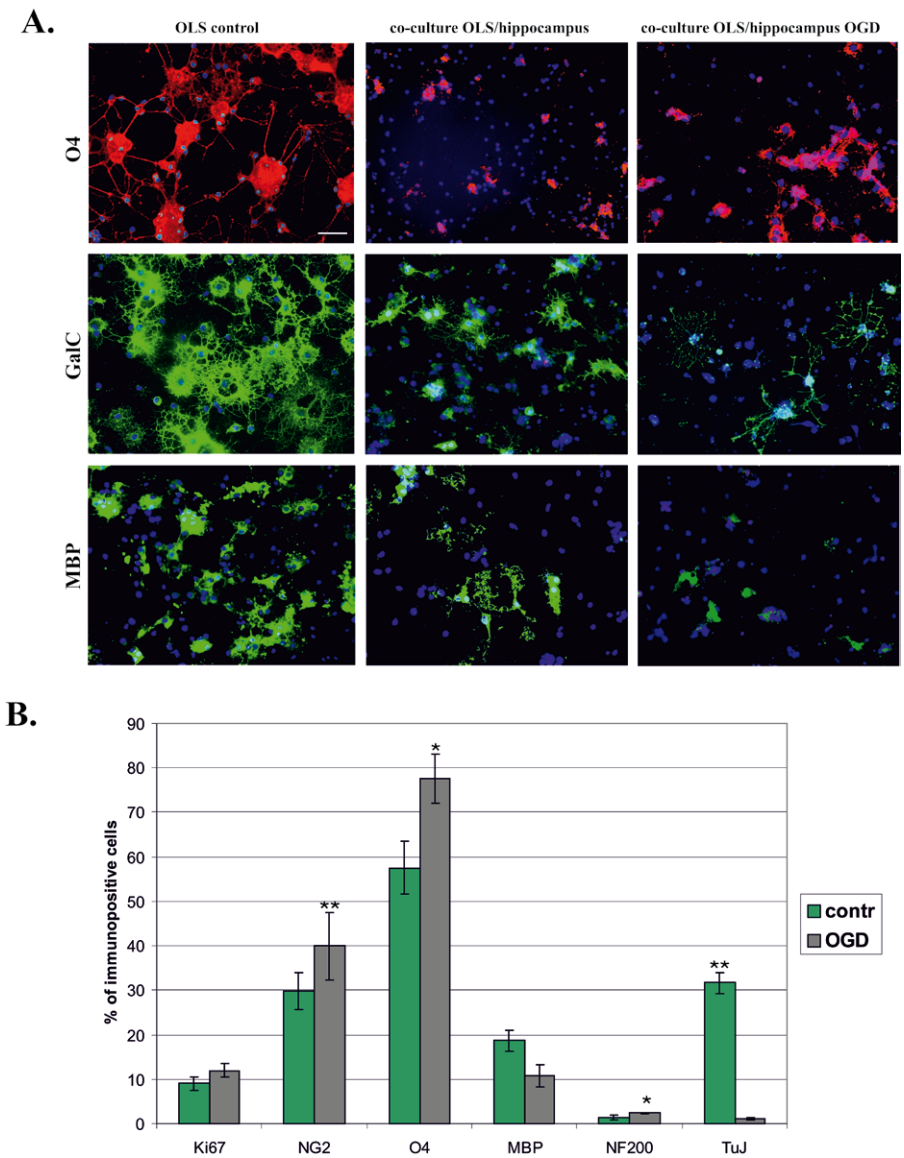

Fig. 2. Differentiation of NG2 precursors in co-culture with either control or OGD – subjected hippocampal organotypic slices during 5 DIV. (A) Immunocytochemical analysis of oligodendroglial cell development: control (left), co-culture with control hippocampal slices (middle) and co-culture with OGD-subjected hippocapmpal slices (right panel). Cell nuclei are visualized by Hoechst 33258 immunostaining (blue). Scale bar is 50 µm. (B) Comparative analysis of oligodendroglial markers relevant to sequential stages of the cell maturation in 5 DIV cell co-cultures (contol hippocampal slices vs. those subjected to OGD procedure). All values on the graphs were expressed as mean ± SEM. Differences were considered significant if: **P*<0.05; ***P*<0.001.

expression characteristic for matured cells was down-regulated in cells differentiating in the vicinity of the spinal cord slices (Fig. 1B). Taken together, the results point to considerable slowdown of oligodendroglial differentiation in co-culture with spinal cord slices when compared both with control cells and those differentiated in the vicinity of hippocampal slices.

Progenitor differentiation in co-cultures with OGD-subjected hippocampal slices

Since the hippocampal microenvironment is very potent in modifying cell capacity to differentiation, subsequently we examined the influence of ischemically injured hippocampal slices on the cell development. The OHC was subjected to temporal oxygen/glucose deprivation to mimic *in vitro* a neurodegenerative insult. After staining with propidium iodide to evaluate cell death, a degree of tissue damage was calculated for each experiment in order to make the comparison and verification of the severity of the insult possible. This allowed us to confirm, that the tissue used for the co-culture experiment was severely injured. The results obtained from the comparison of progenitor differentiation in co-cultures with either intact or OGD-subjected hippocampal slices point to diminished neurogenic effect of the latter, accompanied by delayed maturation of oligodendrocytes (Fig. 2A). The immunocytochemical analysis of sequential stages of development (Fig. 2B) shows that the immature O4-positive cells predominate, although distinctly differentiated branched GalC-expressing oligodendrocytes are also numerous in cells differentiating in vicinity of OGD-subjected slices.

The differences in cell development and amount are also reflected by relative levels of trophic factors, IGF-1 (Fig. 3A) and GDNF (Fig. 3B) expressed by control oligodendrocytes (OLS) and the cells co-cultured with organotypic slices of spinal cord (OLS/OSCC), hippocampus (OLS/OHC) and OGD- injured hippocampal slices, respectively. Data are quantified as fold changes by the $2^{-\Delta\Delta Ct}$ against the β-actin as the housekeeping gene.

Effect of BDNF on fate-decision and differentiation of NG2 progenitors

The potential involvement of BDNF in regulating NG2 cell differentiation has been investigated in dual approach. On one hand, neutralizing (confirmed by ELISA assay) of the endogenous neurotrophin secreted to media in co-cultures resulted in significant slowing down of progenitor maturation, estimated by the expression of the myelin components, and nearly resembled the cell differentiation in co-cultures with the OGD-subjected hippocampal slices. On the other hand, treatment of the developing population of NG2 cells with the exogenous BDNF resulted in the increased

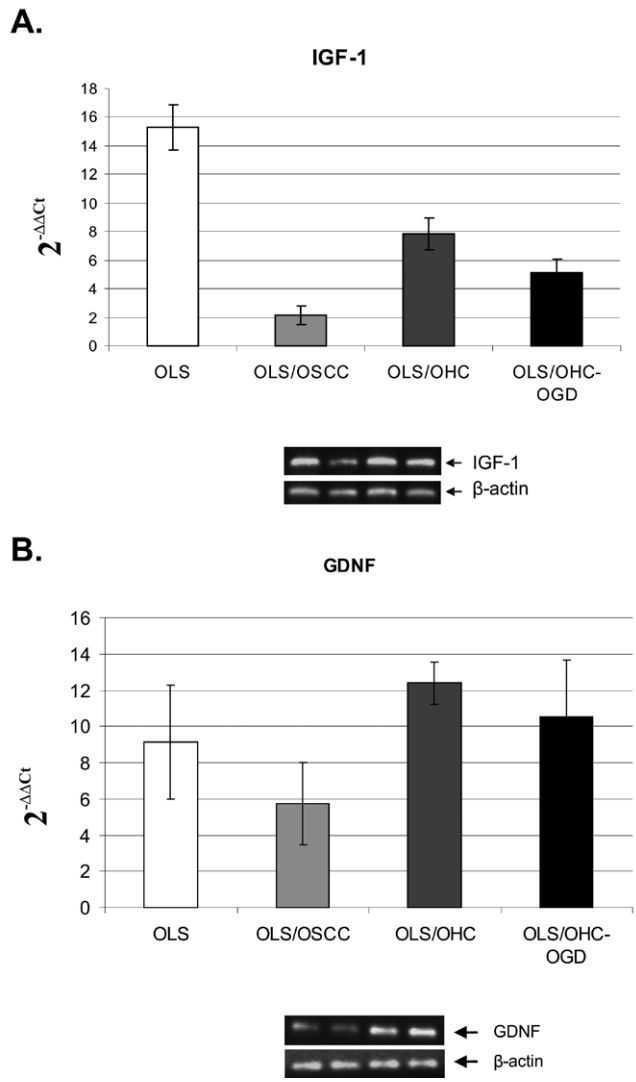

Fig. 3. Relative level of trophic factors (A.IGF-1; B.GDNF) expressed by control OLS and the cells co-cultured with organotypic slices of spinal cord (OLS/OSCC), hippocampus (OLS/OHC) and OGD- injured hippocampal slices, respectively. The observed changes in oligodendroglial factors reflect differences in the cell commitment and growth, depending on the co-culture microenvironment. Data are quantified as fold changes ($2^{-\Delta\Delta Ct}$) against the β-actin as the housekeeping gene.

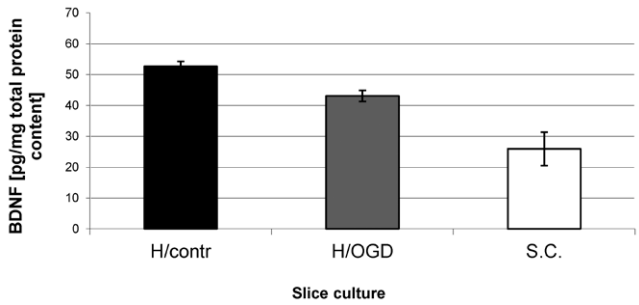

Fig. 4. Concentration of BDNF in the cultured organotypic slices. Differences were considered significant if: **P*<0.05; ***P*<0.001.

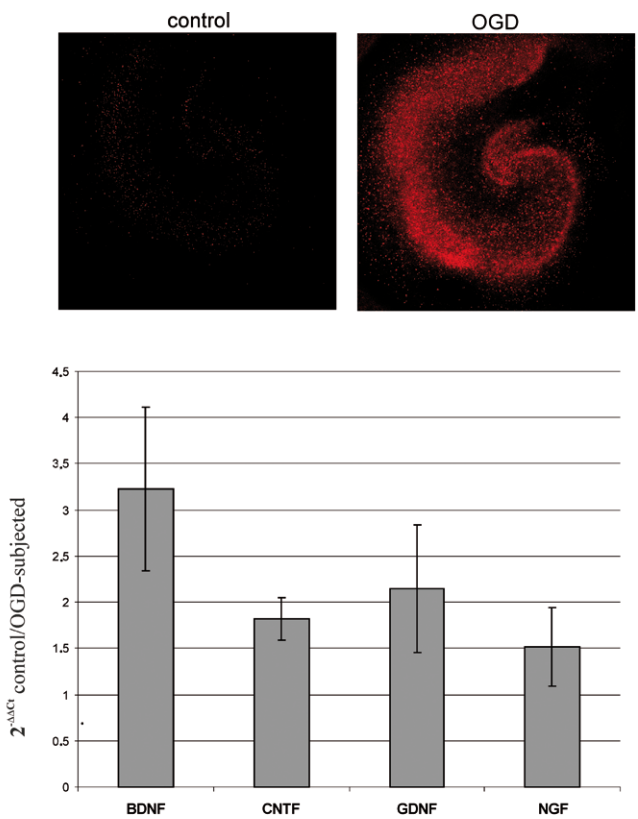

Fig. 5. Decrease in level of trophic factor mRNA observed in hippocampal slices 7 days after OGD procedure. (A) Control and OGD-subjected hippocampal slices stained with PI for cell death evaluation. (B) Relative expression of trophic factors: data are normalized against the β-actin gene as the endogenous control (ΔCt), compared to the intact control slices ((ΔΔCt) and shown as the fold changes ($2^{-\Delta\Delta Ct}$) in the mRNA levels.

(by 8.9 ± 2.31%) number of the MBP-positive cells at 5th DIV. While manipulating the BDNF content in cell culture influenced the maturation of the oligodendroglial progenitors, it however had no significant effect on their neural commitment.

BDNF concentration in CNS-region specific areas revealed significant disparity: in spinal cord slices its level reached only 50% of that estimated in hippocampal slices (25. 95 ± 5.42 pg/mg proteins *versus* 52.69 ± 2.36 pg/mg protein). After OGD procedure, the BDNF concentration drops about 20% (to 41.08 ± 1.78 pg/mg proteins) (Fig. 4). Taken together, it seems that the BDNF might be considered as one of the key factors regulating maturation of the oligodendroglial progenitors.

Down-regulation of trophic factors expression in OGD-injured hippocampal slices

Comparison of normal and traumatized hippocampal slices (Fig. 5A) pointed to striking decrease in expression of the selected factors which are crucial for cell commitment and differentiation: BDNF, CNTF, GDNF and NGF (Fig. 5B). Their relative levels, normalized against β-actin and calculated as fold-changes in comparison to control slices, turned out to be significantly down-regulated, especially in case of BDNF (3.23 ± 0.59).

DISCUSSION

The local microenvironment serves as a reservoir of extracellular signals which regulate the cell physiology and sustain tissue homeostasis. Since neurons and glia are functionally interdependent, numerous molecules must participate in coordinated cell response (Lim and Alvarez-Buylla 1999, Bhat et al. 2001, Allen and Barres, 2005, Goritz et al. 2007). The microenvironment is therefore dynamic, multicomponent and depends on brain regions. Presumably, its composition in gliogenic regions is distinct from that of neurogenic regions owing to both different types of secreted molecules and their reciprocal proportions and interactions. On one hand, this dynamism is essential not only for the regulation of tissue functionality but also makes it possible to respond peculiarly to pathological conditions (Lobjois et al. 2008, Wójcik-Stanaszek et al. 2011a, Zhao et al. 2011). On the

other hand, the specificity of instructive clues cocktail might module the fate choice of mobilized endogenous or transplanted progenitor cells, which should be taken into consideration while planning neurorepair strategies (Nakatomi et al. 2002, Sellers and Horner 2005, Karimi-Abdolrezaee et al. 2010, Kasai et al. 2010, Markiewicz et al. 2011).

Addressing this issue, we examined the differentiation of glia-committed NG2 progenitors in distinct neural microenvironments provided by either hippocampal (neurogenic) or spinal cord organotypic slices (as a model of more gliogenic region). The slices were used to continually condition the co-culture medium for the purpose of examining the potential influence of released soluble factors on the development of co-cultured cells.

Glial progenitors are well-known to quickly maturate *in vitro* (within few days with neither serum nor other stimulating factors having been added) to form oligodendrocytes expressing myelin components (Raff et al. 1983, Trotter and Schachner 1989). Notwithstanding, it is possible to reprogram their fate by adding relatively high concentration of various neuromorphogenes. This observation points to the general susceptibility of NG2 progenitors to influence of biological compounds.

Neurogenesis in hippocampal microenvironment: Comparison of direct contact with effect of indirect co-culture

In our previous study (Sypecka et al. 2009), we showed that the CMFDA-labeled neonatal NG2 progenitors seeded onto the surface of the hippocampal slices migrated within a few hours into the tissue and began to differentiate. After being co-cultured for a week, about one third of the total population of the transplanted cells adopted neuronal phenotypes (TUJ^+, MAP^+). A question arises however, if the observed promotion of neurogenesis is due to cell to cell contact or rather to instructive extracellular diffusible signals. The results obtained in the present study revealed similar susceptibility to the neurogenesis of NG2 progenitors in hippocampal microenvironment in spite of the absence of direct cell contacts. These findings lead to the conclusion, that the orcheastrated influence of instructive paracrine signals in close to physiological concentrations are potent to induce cell reprogramming.

NG2 cell differentiation in distinct tissue microenvironment: hippocampus versus spinal cord

Progenitor commitment and oligodendrocyte maturation require a coordinated spatiotemporal regulation, based on the sequence of highly integrated events (Trapp et al. 1997, Yuan et al. 1998, Ono et al. 2001, Luyt et al. 2006). In highly specialized CNS, different regions are characterized by unique composition of extracellular space. The NG2 cells are known to be scattered throughout the CNS, inhabiting those heterogenous microenvironments, which could modulate either their fate-choice or/and development (Grinspan and Franceschini 1995, Baron et al. 2002, Aguirre and Gallo 2004, Rompani and Cepko, 2010, Wang et al. 2011, Wójcik-Stanaszek et al. 2011b). It is therefore indispensable to learn, how different tissues influence the cell development for the purpose of planning cell replacement therapies.

In our experiments, we showed that the cell commitment and development proceed differently in microenvironments provided by the slices derived from distinct CNS regions. In spinal cord, slow development of glial cells serving for remyelination might be anticipated, while the neurogenesis should be enforced, e.g. by sequential administration of specific pharmacological treatments. Spinal cord microenviroment was also shown to support the proliferative capacity of the NG2 progenitors which is highly desirable for potential tissue restoration. The observed differences in cell commitment and maturation, resulting in the heterogeneity of the populations cultured in various microenvironments, are also reflected by the changes in the expression of oligodendroglial trophic factors: GDNF (Wilkins et al. 2003, Zhang et al. 2009) and insulin-like growth factor 1 (IGF-1) (Wilkins et al. 2001, Ubhi et al. 2010).

Basing on the molecular analysis that revealed over 3 folds decrease in BDNF mRNA level, it amounts in both types of the slices has been measured. The data showed that this protein content is doubled in hippocampal slices, which presumably significantly contributes to the microenvironment heterogeneity and affects biology of oligodendrocytes (Van't Veer et al. 2009, VonDran et al. 2011). Examination of the effect of BDNF alone on the neuronal commitment of NG2 progenitors suggests that it might be rather one of the players in the orchestrated, synergistic influence of various signals creating the local microenvi-

ronment. This neurotrophin however seems to be the potent regulator of the region-specific maturation of the oligodendroglial progenitors and therefore could be used in situ to prevent apoptosis and to control cell differentiation (Nakajima et al. 2010, Rosenberg et al. 2006, Chen et al. 2013).

NG2 cell development in traumatized microenvironment

Oligodendrocytes and their progenitors are extremely vulnerable to cytotoxic compounds (Sypecka, 2003, Deng et al. 2004, Buzanska et al. 2009, Kuzhandaivel et al. 2010) and are known to be injured in many acute and chronic conditions (Dewar et al. 2003, Back et al. 2007, Watzlawik et al. 2010, Watson and Yeung 2011, Boscia et al. 2013). Their susceptibility to insults (including those resulting from disturbances in local tissue homeostasis like hypoxia) is highiest in perinatal period when oligogliogenesis is the most intense (Sypecka 2003, French et al. 2009, Buser et al. 2012). It seems however that embryonic progenitors or those generated in early postnatal period still have some properties of neural stem cells (Zheng et al. 2006, Ju et al. 2012) and their fate-choice might be modulated by local endogenous (or possibly) exogenous signals (Jin et al. 2012, Sypecka 2011, Tuinstra et al. 2012). In this regard, the hope-rising observation coming from the presented study concerns progenitor development in pathological conditions caused by OGD insult. Although neurogenic effect exerted by the control hippocampal slices is significantly diminished and the oligodendroglial maturation is visibly slowed down, nonetheless cell development progresses in such traumatized microenvironment. Moreover, the NG2 progenitors are still able to proliferate and to adopt different neural phenotypes, most probably thanks to trophic stimuli provided by the survived neurons. These processes might be even enhanced by the injury-evoked increase in the expression of selected neurotrophins that govern the cell cycle and development

CONCLUSION

The presented work show a lineage plasticity of the NG2 progenitors, depending on the tissue local microenvironment, which might be beneficial for the strategies promoting the CNS repair based either on the endogenous cell recruitment or transplantation.

ACKNOWLEDGEMENTS

We thank Mrs. Hanna Winiarska and Mr. Lukasz Strojek for excellent technical assistance. This work is supported by Polish Ministry of Science and Higher Education grant 0345/B/P01/2010/38 and 05728/B/NZ4/2011/01.

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