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Oxygen-glucose deprivation promotes gliogenesis and microglia activation in organotypic hippocampal slice culture: Involvement of metalloproteinases

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Organotypic hippocampal cultures are used as an alternative model for studying molecular mechanism(s) of neurogenesis after combined oxygen-glucose deprivation (OGD) mimicking ischemic conditions. The aim of the present work was to investigate the effect of OGD on stem/progenitor cells proliferation and/or differentiation in the hippocampus. Our attention was primarily focused on the relationship between neurogenesis-associated processes and activity of matrix metalloproteinases (MMPs). Cell proliferation was detected by using BrdU incorporation. Newly generated BrdU (+) cells were identified by labeling with specific cell markers. In order to check the activity and localization of MMPs we conducted *in situ* zymography in conjunction with immunohistochemistry. In our experimental conditions OGD-insult followed by 24 h of recovery caused the damage of neuronal cells in CA1. At 1 week cell death appears all over the hippocampus. We found that expected stimulation of endogenous neurogenesis fails as a source of compensation for the lost neurons in OGD-treated cultures. The modulation of culture microenvironment after ischemia favors the dominant proliferation of glial cells expressed by the enhancement of newly-generated oligodendrocyte progenitors. In addition, during our study we also detected some BrdU labeled nuclei encapsulated by GFAP positive processes. However, the majority of BrdU positive cells expressed microglial specific stain, particularly pronounced in CA1area. The OGD-promoted responses involved activation of metalloproteinases, which matches the progression of gliogenesis. On the other hand, the high activity of MMPs associated with microglial cells implicate their involvement in the mechanism participating in OGD-induced cell damage.

Key words: ischemia *in vitro*, hippocampus, neurogenesis, oligodendrocytes, astrocytes, matrix remodeling

INTRODUCTION

Several data published in the last decade confirmed that ischemia produced enhanced neurogenesis in neuroproliferative regions of the adult rodent brain, including subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Arvidsson et al. 2002, Tonchev et al. 2003, Jablonska and Lukomska 2011, Wojcik-Stanaszek et al. 2011). Ischemia-induced cell proliferation and neurogenesis was considered as compensatory mechanism of neuronal loss. It is therefore tempting to speculate, that the amplification of self-repair mechanism might in the future become a therapeutic modality in brain disorders.

Recent efforts have utilized organotypic hippocampal slices (OHC) to investigate mechanisms and treatment strategies for brain disorders. Studies using organotypic hippocampal slice cultures have predominantly focused on "ischemic like" oxygen-glucose deprivation (OGD), which might mimic the *in vivo* situation, including selective vulnerability of pyramidal neurons in the CA1 sector of the hippocampus (Noraberg et al. 2005). Organotypic cultures offer the advantages of tissue-specific cell connections, preservation of local neuronal circuits with the appropriate innervation patterns, but exclude complex parameters which are present in animal models, such as blood supply and blood-brain barrier (Buchs et al. 1993). Since OHCs combine the accessibility of an *in vitro* system and the retained three-dimensional structure of the

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respective tissue found *in vivo*, it can serve as a model for the investigation of cell proliferation/differentiation in the CNS. Till now little is known about the effect of oxygen-glucose deprivation on neurogenesis in OHCs. Furthermore, the available data are inconsistent

The identification of mechanism(s) that promote the proliferation of progenitors and differentiation into the phenotype of damaged neuronal cells has become particularly relevant to the development of stem cell-based therapies. Any effective cell replacement therapy as well as efficient stimulation of neurogenesis will require the basic understanding of mechanism governing stem cells proliferation and efficient differentiation into a particular neural phenotype. Increasing data indicate the importance of the expression and production of both adhesion molecules and extracellular matrix components, which contribute to the formation of a unique environment which may be instructive or permissive to neurogenic-associated processes (Bovetti et al. 2007). In this context enzymes with ability to modify the extracellular matrix are particularly interesting (Ethel and Ethel 2007).

MMPs are secreted or membrane-bound endopeptidases, responsible for the processing of a variety of pericellular substrates including extracellular matrix proteins, cell surface receptors, cell adhesion molecules and growth factors (Rivera et al. 2010). Whereas MMPs, particularly gelatinases – MMP-2 and MMP-9, have been mostly investigated in the context of detrimental roles in brain ischemia (Rivera et al 2002, Zalewska et al. 2002, Gasche et al. 2006) several studies showed that they could be involved in the neurogenic response of adult neural stem/progenitor cells in the ischemic brain (Barkho et al. 2008, Jablonska and Lukomska 2011, Wojcik-Stanaszek et al. 2011). MMPs are expressed abundantly in neural stem cells isolated from human central nervous system (Frolichsthal-Schoeller et al. 1999) and their involvement in the proliferation and differentiation of neural precursor cells has been confirmed (Mannelo et al. 2006). Furthermore, Lu and coauthors (2008) showed that up-regulation of MMP-2 and MMP-9 in the neurogenic area of the dentate gyrus was compatible with the peak of post-ischemic neurogenesis in adult primate brains. Altogether, these data strongly suggest the participation of MMPs in ischemic injury repair, favoring the migration of precursor stem cells from neurogenic into injured sites to replenish cell lost.

Despite the ever-growing information concerning the engagement of MMPs in neurogenesis-associated processes *in vitro* and *ex vivo* after brain ischemia, the response of MMPs to OGD in organotypic hippocamal slices is still unknown.

This prompted us to investigate the effect of OGD on the proliferation and/or differentiation of stem/progenitor cells in hippocampal slice cultures. Our attention was primarily focused on the potential relationship between proliferation of neural stem cells and/or differentiation and activity of MMPs.

METHODS

Organotypic hippocampal culture (OHC)

The experiments carried out were approved by the Local Commission of Ethics for Experiments on Animals. Organotypic hippocampal slice cultures (OHCs) were prepared according to the method of Stoppini (Stoppini at al. 1991). Pups of 7–9-day- old Wistar rats were sacrificed by decapitation and the hippocampi were rapidly dissected and transversely sliced at 350-μm thicknesses using a McIlwain tissue chopper. The slices were carefully transferred onto permeable (0.4 μm porous) Millicell-CM (Millipore) membranes (4 slices on each) and deposited in 6 well-plates (Nunc). Slices were cultured for 2 days at 35°C, 5% CO_2 in fully humidified atmosphere in serum-based growth medium (0.9 ml/well) containing 50% DMEM, 25% HBSS, 17mM HEPES, 5 mg/ml glucose, 2 mmol/l L-glutamine, 25% horse serum, 1% amphotericin B and 0.4% penicillin-streptomycin (pH 7.2). For the following four days the serum content was gradually lowered and at 6-day *in vitro* (DIV) cultures were transferred to a serum-free medium based on DMEM/F12 with 25% HBSS, 17 mM HEPES, 5 mg/ml glucose, 2 mmol/l L-glutamine, 25% Neurobasal-A, 1% B27, 1% amphotericin B and 0.4% penicillin-streptomycin and kept in a tissue culture incubator at 35°C in 5% CO_2. OHC were selected with propidium iodide (PI, 0.5 μM; Sigma) before the experiment to exclude damaged cultures. PI was added to the culture medium for 24 h. Only PI-negative OHC were selected for further experiments using fluorescence microscope (Axiovert 25, Carl Zeiss, Germany).

Oxygen-glucose deprivation (OGD)

Oxygen glucose deprivation was used as an *in vitro* model of cerebral ischemia. OGD was performed on 7

days old culture (DIV). The membranes with hippocampal slices were transferred to a 6 well-culture plates with 0.9 ml of Ringer solution containing 10 mM mannitol instead of glucose (OGD medium) saturated with a mixture of 95% N_2/5% CO_2 by 10 minutes flushing. The cultures were then placed in humidified oxygen-free chamber for 40 minutes at 35°C. OGD was terminated by returning the slices to a normoxic conditions and cultured further in serum-free medium for 1, 3 and 7 days (8, 10, 14 DIV). Control slices were kept under normoxic atmosphere in the glucose-containing Ringer solution. Proliferating cells were detected by using DNA replication marker 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich), which was added to the incubation medium (20 μM final concentration) immediately after exposure to OGD (7 DIV) and was present during the entire time of incubation. The medium was changed 3 times a week. Each experimental group consist 12–16 cultured slices from 6 independent rats.

Evaluation of cell damage

Propidium iodide was used as a marker of cellular damage in control and in OHC exposed to OGD (Pozzo Miller et al. 1994). Immediately after the deprivation of glucose and oxygen and throughout the entire culture period (8, 10 and 14 DIV) the sections were incubated for 2 hours in 35°C in serum free medium containing 10 μM propidium iodide. The high resolution fluorescent images were acquired by confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany) and photomicrographs of sections were imported into ImageJ 1.46 software. Intensity of fluorescence was evaluated in the entire CA1 and DG area.

Immunohistochemistry

The following primary antibodies (source and final dilution) were used for immunostaining: sheep polyclonal anti-BrdU (Abcam, 1:500), mouse monoclonal anti-BrdU (Santa Cruz Biotechnology, 1:100), mouse monoclonal anti-neuronal β-tubulin III (Sigma, 1:500), mouse monoclonal anti-neuronal nuclear antigen (NeuN; Milipore, 1:500) mouse monoclonal anti-MAP-2 (Sigma, 1:500), mouse monoclonal anti-neurofilament 200 (NF-200; Sigma, 1:500), rabbit polyclonal anti-GFAP (Cappel, 1:200), goat polyclonal anti-Iba1 (Abcam, 1:600), mouse anti-rat ED1 CD68 (Serotec, 1:100), rabbit polyclonal anti-NG2 Chondroitin Sulfate proteoglycan (Milipore, 1:200). Primary antibodies were visualized using either Alexa Fluor 488 (green) or Alexa Fluor 546 (red) (Molecular Probes, 1:500).

At the scheduled incubation time cultures were washed with 0.1 M phosphate- buffered saline (PBS) for 15 minutes, fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 30 minutes and rinsed three- times for 5 minutes in PBS.

For BrdU immunostaining, DNA was first denaturated in 2N HCl at 37°C for 60 minutes. Then hippocampal slices were incubated in 0.1M sodium tetraborate (pH 8.5) for 15 min, blocked for 60 min in 10% normal goat or donkey serum diluted in PBS with 0.25% Triton X-100, and incubated overnight with primary antibody at 4°C. For the removing of unbound antibody sections were washed three times with PBS, then incubated for 60 min with FITC- tagged secondary antibody at room temperature and coverslipped using mounting media. Double-label immunohistochemistry was achieved by co-incubation with anti-mouse, anti-goat, anti-sheep, and anti-rabbit primary antibodies conjugated to distinct fluorophores respective for each species of primary antibody.

The phenotypes of BrdU positive cells were determined with: beta-tubulin III, NF-200, NeuN (neuronal markers), GFAP (astrocytic marker), NG-2 (marker of oligodendrocyte progenitors) ED-1 (marker of microglia). After BrdU staining the hippocampal sections were incubated overnight (4°C) with primary antibodies. After rinsing in PBS, slices were exposed for 1 h to secondary antibodies (room temperature). Negative controls were processed in the same manner with the primary antibodies omitted. Cell nuclei were visualized by incubating with 5 μm Hoechst 33258 (Sigma). Double labeling to detect the expression of phenotypic markers by BrdU expressing cells was verified using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany). The number of BrdU-positive as well as double-stained cells in the DG and CA1 area was assessed in an average of 3-5 hippocampal slices per animal by manual counting area 0.38 mm^2.

In some sections co-labeling was confirmed by z-stack analysis.

In situ zymography

In order to localize activity of metalloproteinases (MMP-2 and MMP-9) within control and ischemic hippocampal slices we performed in situ zymography according to previously described method (Rivera at al. 2002, Wójcik-Stanaszek at al. 2011). Hippocampal slices were incubated for 3 h at 37°C in a humid dark chamber in reaction buffer containing 50 µg/ml of FITC-labeled DQ-gelatin (Invitrogen Molecular Probes, Eugene, OR) that is quenched intramolecularly. Gelatin-FITC cleavage by tissue metalloproteinases (gelatinases) releases peptides whose fluorescence is representative of proteolytic activity. After incubation with substrate the sections were rinsed in PBS, fixed for 30 min in cold 4% PFA, mounted in fluorescent mounting medium (Dako) and analyzed using confocal microscope (LSM 510, Carl Zeiss, Germany). The intensity of fluorescence was evaluated by using ImageJ 1.46 software. All images subjected to direct comparisons were captured at the same exposure and digital gain settings to eliminate confounds of differential background intensity or false-positive fluorescent signals across sections.

In the next set of sections double fluorescent labeling was performed in order to identify the cell types expressing gelatinolytic activity. After zymography the slices were rinsed in PBS at pH 7.4 and fixed for 30 minutes in 4% PFA. After rinsing in PBS floating slices were preincubated in a blocking solution (10% normal goat serum in PBS containing 0.25% Triton X-100) for 60 minutes and then incubated overnight at 4°C with proteins specific for neurons (NF-200 and MAP-2), astrocytes (GFAP) or microglia (Iba1 and ED1). In addition, some sections were immunostained with BrdU antibody according to the procedure described above.

Image analysis and quantification

Image analysis of BrdU and double immunofluorescent labeling was performed by confocal laser scan microscopy (LSM 510, Carl Zeiss, Germany) using 20× objective. A helium-neon laser (543 nm) was utilized in the excitation of Alexa Fluor 546, while an argon laser (488) was applied in the excitation of FITC. All images subjected to direct comparisons were captured at the same exposure and changed to gray scale to eliminate confounds of differential background intensity or false-positive immunoreactivity. Using ImageJ software, the region of interest (CA1 and DG) was outlined and the total mean fluorescence intensity was recorded and compared to control staining. In some sections co-labeling was confirmed by z-stack analysis.

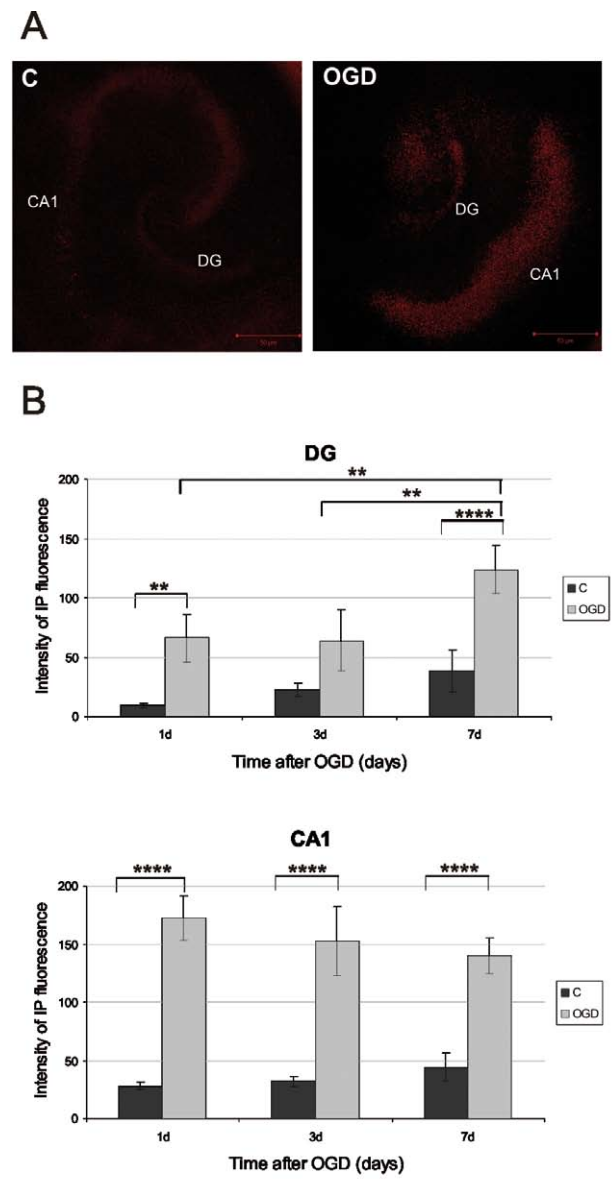

Fig. 1. Time course of neuronal cell damage in organotypic hippocampal culture after OGD. (A) Fluorescent light images show PI uptake under control (c) and at 24 h after OGD. (B) Graphs show the intensity of PI fluorescence in DG and CA1 area under control (black bars) and at different time points after OGD (1, 3 and 7 days) (grey bars). Values represent the means ± SEM of six animals per time point. One-way ANOVA and Bonferroni test: $^{**}P<0.01$ and $^{****}P<0.0001$ indicate a statistically significant difference.

Statistical comparisons among groups were done using ANOVA with *post-hoc* Bonferroni test. All data were presented as mean ± standard error of the mean (SEM). Statistical significance was deemed to be present if $P<0.05$.

RESULTS

Time course of cell damage after OGD

The time-course of cell damage in the hippocampal slice cultures was evaluated by cellular PI uptake at specific time points after the ischemic insult (1, 3 and 7 days). The results showed that a brief ischemic injury (40 minutes OGD) followed by a reperfusion under normoxic conditions caused a significant damage of neuronal cells, particularly in CA1 pyramidal layer (Fig. 1A, B). At 24 hours of reoxygenation the inten-

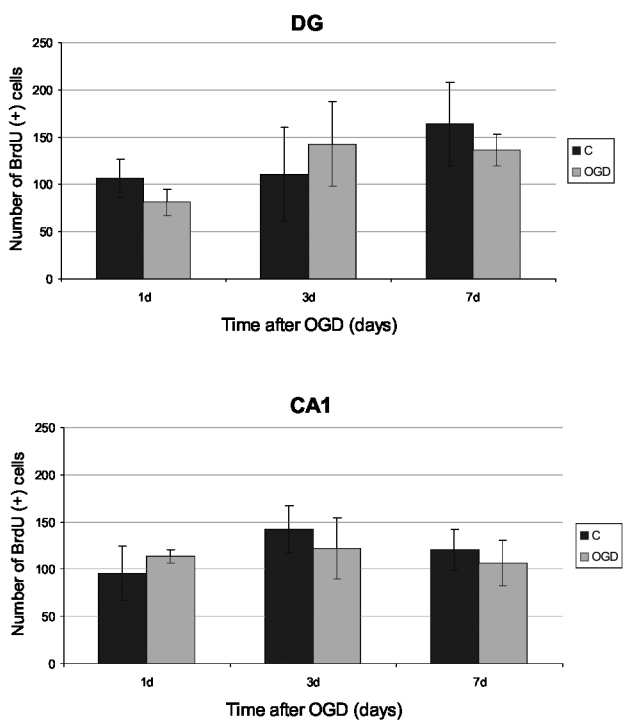

Fig. 2. Time-course of cell proliferation in organotypic hippocampal culture under control and after OGD. OHC were exposed to 40 min OGD. BrdU (15 µg/ml) was present during the entire time of incubation Cell proliferation was detected by BrdU immunostaining. Graphs show the number of BrdU-labeled nuclei quantified in the DG and CA1 area 0.38 mm^2. Values represent the means ± SEM of six animals per time point. One-way ANOVA and Bonferroni test did not indicate significant differences in BrdU labeling between control (black bars) and OGD-treated slices (grey bars).

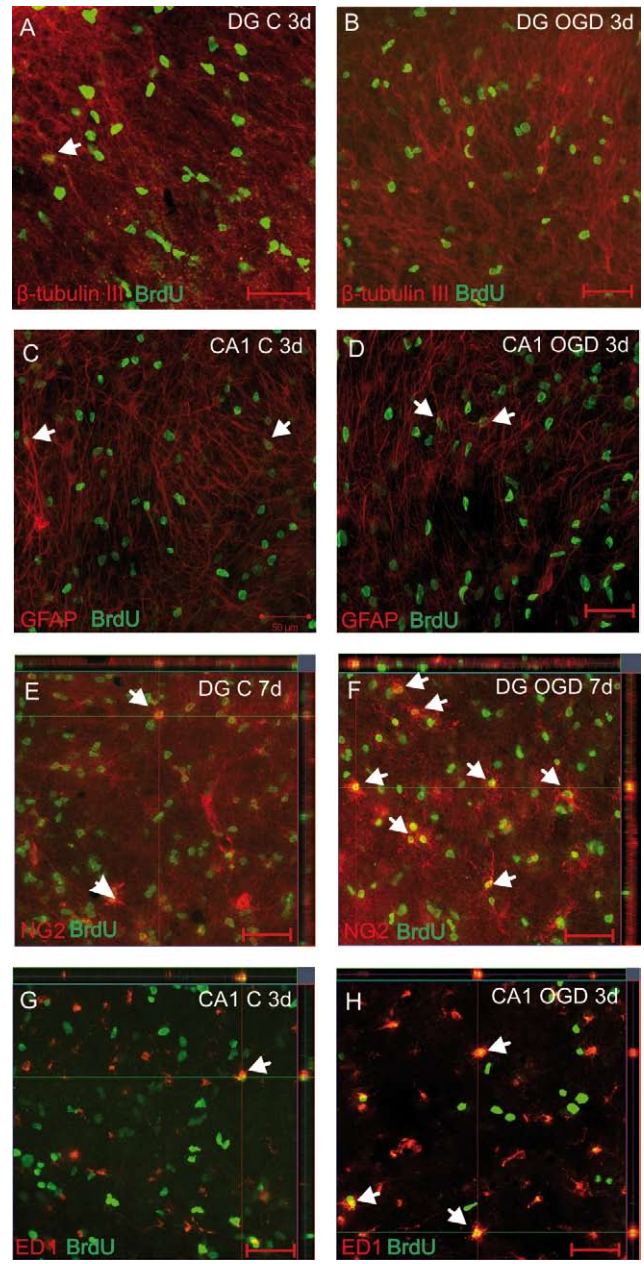

Fig. 3. Identification of proliferated cell types in organotypic hippocampal culture under control and after OGD. OHC were double-stained with BrdU immunoreactivity (green) and young neuron-specific beta III tubulin (A, B), astrocyte-specific GFAP (C, D), oligodendrocyte-specific NG2 (E, F) and microglia specific ED1 (G, H) markers (red). After OGD (right panel) some BrdU-labeled nuclei encapsulated by GFAP positive processes in CA1 was barely higher than in control (left panel). Similarly, the high number of the BrdU positive cells expressing ED1 was clearly pronounced in CA1, while BrdU/NG2 positive cells were located mainly in DG. Photomicrographs are representative of observations made from 6 animals per time point. Scale bar is 50 µm.

sity of PI fluorescence in CA1 was 7-fold higher, compared to the control culture, and remained on the similar level up to 7 days of reperfusion. OGD- induced cell damage was not restricted to CA1. Remarkable elevation of PI uptake was observed also throughout the dentate gyrus region, with the peak of fluorescence seen 7 days after ischemic insult. In this time point the intensity of signal was enhanced 3-fold *versus* the respective control. There was negligible cells damage in control, compared to that seen in OGD-treated cultures. The baseline of PI fluorescence is probably due to the spontaneous degeneration of some neurons, increasing with the subsequent culturing phase.

Cell proliferation

Cell proliferation in organotypic hippocampal slices was studied at different time of reoxygenation (1, 3 and 7 days) after deprivation of glucose and oxygen. For this purpose cultures exposed to OGD as well as intact cultures were incubated afterwards with culture medium containing BrdU (15 μg/ml) for up to 7 days. The number of newly-generated cells was determined in the DG and CA1 structure by monitoring the incorporation and subsequent immunohistochemical detection of BrdU. As depicted in the graphs (Fig. 2), a number of cells incorporating BrdU are seen within the culture incubated in normoxic conditions. During the entire time dividing cells were almost evenly distributed among both structures – DG and CA1. Exposure to OGD did not change the pattern of BrdU labeling however, 3 days after OGD an outlined inclination to increased number of dividing cells was observed in DG. As depicted in Figure 8 (arrowhead) BrdU labeled cells occasionally appeared as pairs being apposed each other.

To further characterize the fate of dividing cells, hippocampal slices were double stained for BrdU and known neuronal antigens – betaIII-tubulin (for newly born neurons), NF-200 (detected in immature as well as in mature neurons) and NeuN (for developed neuronal cells), GFAP for astrocytes, and NG2 for precursors of oligodendrocyte. To identify newly-generated microglia anti-BrdU antibody was used in combination with ED1 and Iba1.

Under close inspection of confocal image we have found only a few BrdU cells expressing betaIII-tubulin in the DG of control OHC (Fig. 3A). In contrast, we did

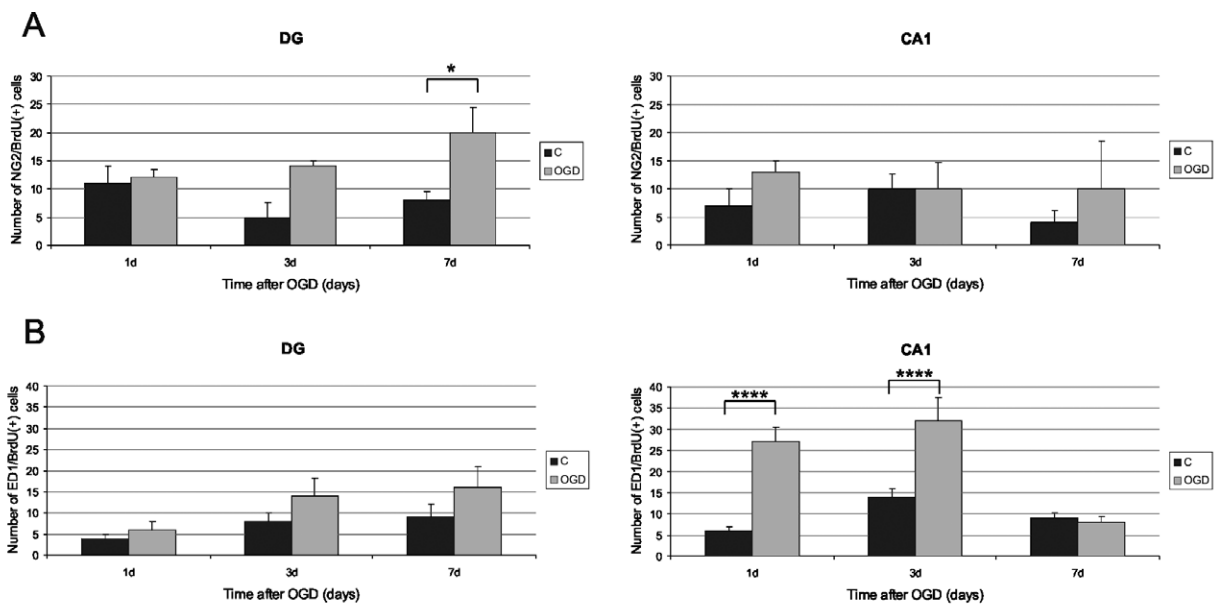

Fig. 4. Proliferation of oligodendrocyte progenitors and microglial cells in hippocampal slice cultures after OGD. Graphs show the number of BrdU/NG2 (A) and BrdU/ED1 (B) labeled cells quantified in the DG and CA1 area 0.38 mm^2. Values represent the means ± SEM of six animals per time point. One-way ANOVA and Bonferroni test indicate significant differences in double labeling between control (black bars) and OGD-treated slices (grey bars). *P<0.05 and ****P<0.0001.

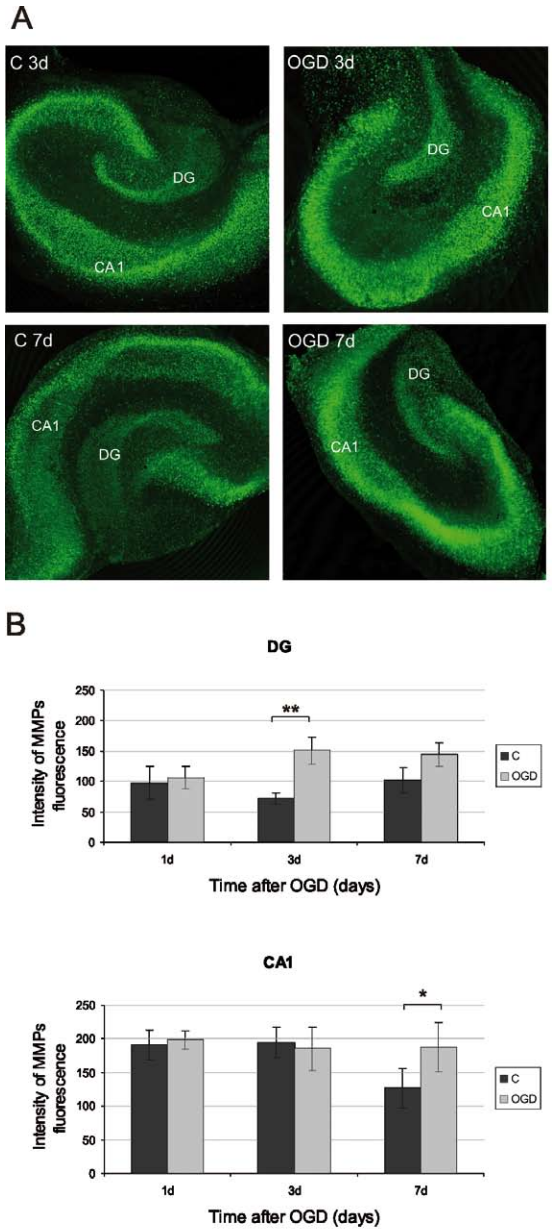

Fig. 5. Activity of metalloproteinases (MMP-2 and MMP-9) in organotypic hippocampal culture after OGD. (A) Confocal photomicrographs showing *in situ* zymography in the control (left panels) and at 3 and 7 days after exposure to OGD (right panels). (B) Graphs show quantitative analysis of MMPs activity in DG and CA1 expressed by the intensity of fluorescence signal. All images subjected to direct comparisons were captured at the same exposure and digital gain settings. Note the increase of MMPs activity after OGD (grey bars) in both hippocampal subfields, as compared to the control (black bars). Values represent the means ± SEM of six animals per time point. One-way ANOVA and Bonferroni test: **P*<0.05 and ***P*<0.01 indicate statistically significant differences.

not detect cells expressing this antigen in cultures treated to OGD (Fig. 3B). Furthermore, we did not find neither co-staining with NF200 nor co-localization with NeuN, the marker specific for mature neuronal cells. It seems therefore obvious, that in our experimental conditions BrdU labeled cells do not acquire phenotypes of mature neurons.

Ischemic insult did not affect markedly the number of GFAP-positive cells, compared to untreated cultures. A barely higher number of BrdU labeled nuclei surrounded by GFAP-positive processes, were observed at 3 and 7 days of recovery after OGD compared to the respective control (Fig. 3C, D). GFAP immunostaining revealed multipolar feature with processes extending through a wide area of the tissue.

A low number of newly generated double stained cells BrdU/NG2 types presenting the basal level of oligogliogenesis appeared in control OHC. These cells were almost evenly distributed in DG and CA1. In response to OGD the number of BrdU/NG2 positive cells increased. The enhancement of newly-born OPCs was most pronounced in DG, especially at longer time of reperfusion – two-fold more oligodendrocyte progenitors were found seven days after ischemic insult (Fig. 3E, F, Fig. 4A).

Many BrdU positive cells also include microglia. The use of anti-BrdU antibody together with microglial specific stain demonstrates a remarkable increase of proliferating microglial cells after OGD, compare to normoxic conditions. A most prominent elevation of new microglia was noted in CA1 area at 1–3 days after the ischemic insult (8–10 DIV), when above 40% of the BrdU labeled cells expressed the microglial marker ED1, compared to about 10% in untreated culture (Fig. 3G, H, Fig. 4B). Thereafter their number appeared to return to the control level. An elevation of proliferated cells was observed also in DG, however, the peak was seen between 3–7 days (10–14 DIV) (19 *versus* 5 cells, respectively) and reached about 10% of the total BrdU-positive cells. The same results were observed with Iba1.

The effect of OGD on the activity of MMPs in the organotypic hippocampal slices

In order to check whether changes in net MMP activity accompanied cells proliferation in hippocampal organotypic culture we used *in situ* zymography. The proteolytic activity of MMPs was determined in

the DG and CA1 structure by evaluation of fluorescence intensity. Almost constant basal activity, with the more intensive fluorescence signal in CA1 region, was maintained during the entire incubation period in control slices. Exposure of culture to OGD resulted in an elevation of gelatinases activity, comparatively to normoxic conditions (Fig. 5A, B). In the DG significant increase of fluorescence signal was observed between 3–7 days of recovery (10–14 DIV). At the same time the intensity of fluorescence presents increasing tendency in CA1 area, compared to the control, however statistical significance was noticed only at 7 days after ischemic insult (14 DIV). It is worth to point out that the increment in activity coincides with proliferation of oligodendrocytes and microglia.

In order to identify the cell types expressing gelatinolytic activity *in situ* zymography was followed by double fluorescent labeling with proteins specific for either neurons (NF200), astrocytes (GFAP), and microglia (ED1 and Iba1). Activity of MMPs manifested itself in all analyzed neural cells (Figs 6 and 8). A strong fluorescence signal was observed in Hoechst counterstained nuclei. However, proteolytic activity was also seen in the cytoplasmic compartment and outside cell bodies. In the ongoing work we did not include the microphotographs demonstrating co-staining of NG2 with MMPs. The appropriate figure has been presented in our previously published report (Sypecka et al. 2009, Wojcik-Stanaszek et al. 2011).

Activity of metalloproteinases associated with microglial cells was mostly noticeable in CA1 area (Fig. 6D, Fig. 7). Numerous of them show simultaneous labeling with BrdU (Fig. 8).

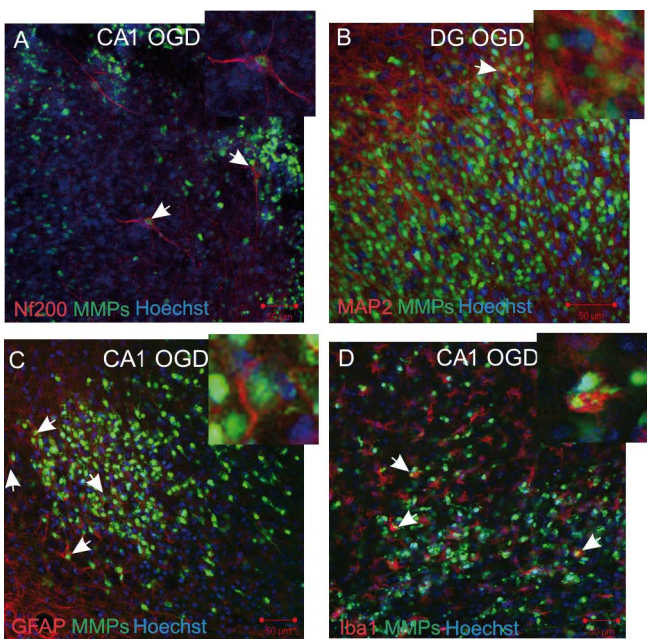

Fig. 6. Distribution of metalloproteinases (MMP-2 and MMP-9) in organotypic hippocampal culture after OGD. Confocal photomicrographs show co-staining of MMPs (*in situ* zymography, green) with neuronal markers (red): NF200 (A) and MAP2 (B); astrocytic marker GFAP (C), and microglia marker Iba1 (D). Note the presence of activity in all investigated cell types, however is most pronounced in GFAP- and Iba1-labeled cells (C,D). MMPs are seen in the cytoplasm, in nuclei and extracellularly. Photomicrographs are representative of observations made from 6 animals per time point. Scale bar is 50 µm.

DISCUSSION

The current results demonstrate that exposure of organotypic hippocampal cultures to oxygen-glucose deprivation does not lead to the stimulation of neurogenesis. Quantitative analysis of cell proliferation indicates almost equal number of proliferating cells in intact as well as in OGD-treated culture. Furthermore, BrdU-positive cells remain on the same level in both investigated hippocam-

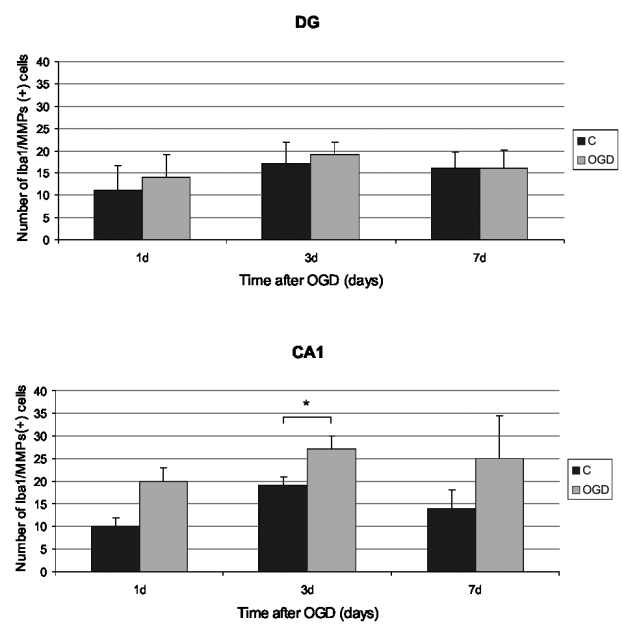

Fig. 7. Expression of metalloproteinases in microglial cells in organotypic hippocampal culture after OGD. Graphs show the number of Iba1/MMPs double labeled cells quantified in the DG and CA1 area 0.38 mm^2. Values represent the means ± SEM of six animals per time point. One-way ANOVA and Bonferroni test indicate significant differences in double labeling between control (black bars) and OGD-treated slices (grey bars). *P<0.05.

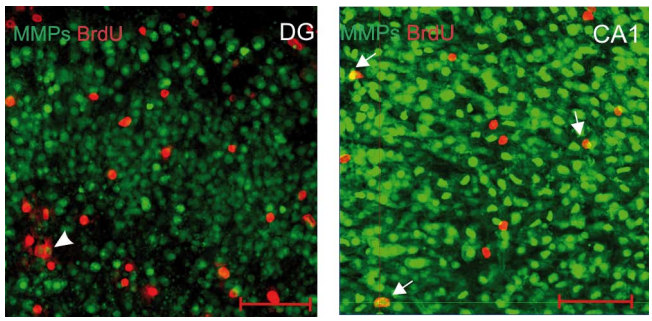

Fig. 8. Localization of MMPs in BrdU labeled cells. Confocal photomicrographs show co-staining of MMPs (*in situ* zymography, green) with BrdU (red) in hippocampal subfields. Note the appearance of double BrdU/MMPs staining in dividing cells (as a pairs being apposed each other) (arrowhead in left panel). Scale bar is 50 μm.

pal regions which demonstrate various susceptibility to ischemic injury (Kirino 1982). Indeed, we have detected appearance of a few BrdU-labeled cells expressing betaIII tubulin only in control OHC at 10 days *in vitro*. The presence of these cells indicates that a small number of progenitor cells commit to neuronal differentiation. However, during the time of our experiment we did not observe co-localization of BrdU with NeuN, the marker specific of developed neurons. Probably these newly-generated young neuronal cells need longer time for maturation or undergo programmed cell death shortly after generation. According to Namba and coauthors (2007) neural precursor cells lost their neurogenic capacity after 7 days in control culture. In contrast, Kamada and colleagues (2004) using a retrovirus vector transduction method found that newly divided cells were able to proliferate for at least four week culture period. The above described findings are related to different experimental setup.

In general our results are in agreement with previous report published by Chechneva and others (2006). They demonstrated a marked increase of BrdU+ cells in CA1 as well as in DG after OGD. However, the acceleration of proliferation has been noted only during the first 24 h after ischemia. In our study 24 h has been chosen as the first time point for monitoring the incorporation of BrdU. By then in our as well as in experiment performed by Chechneva and colleagues (2006) no difference in the number of BrdU+ cells between CA1 and DG was observed. Moreover, the number of BrdU/ betaIII-tubulin positive cells in the area dentata did not show significant difference between control and OGD-treated culture (Strassburger et al. 2008). Interestingly, OGD-induced significant increase of neuroblasts (BrdU/DCX) and newly-generated young neurons (BrdU/beta III tubulin positive) was noted in adjacent to hippocampus post paraventricle identified as the second, beside DG, neuroproliferative zone (Chechneva et al. 2005). It needs, however, to be pointed out that whereas DCX is specific for identification of neuroblasts the use of beta III tubulin as a marker of unmature neuronal cells became questionable. In agreement with our finding, Chechneva and coworkers (2006) did not find the presence of matured neurons after OGD.

From the above, it follows that in our experimental conditions the expected endogenous neurogenesis fails as a source of compensation for the lost neurons in OGD insulted culture and that the hippocampus merely displays gliogenesis.

This finding is consistent with the accepted view that the modulation of post-ischemic hippocampal culture microenvironment after OGD favors the dominant proliferation of glial, rather than neuronal precursors, thus reducing neurogenesis (Kempermann and Neumann 2003). In line with this, our current results revealed the increased number of BrdU-positive cells expressing oligodendrocyte progenitor marker (NG2) after OGD insult, compared with the untreated culture. This observation has found strong support in experiments performed previously in our laboratory (Sypecka et al., unpublished results). Oligodendrocyte precursors in conditions of our experiment (incubation in serum-free medium) most probably differentiate into mature oligodendrocytes (Raff et al. 1983).

Until recently it have been generally accepted that OPC served as a reservoir of developed oligodendrocytes, which contribute to axonal myelination (Sypecka 2011). However, the detection of their presence within the unmyelinated axons of DG granule cells and active proliferation in response to neuronal activity suggest, according to Matsumoto and coauthors (2011), their participation in modulation of synaptic plasticity for the hippocampal function. Then, one has to consider that the increased number of OPCs in non-neurogenic DG area in OGD-treated hippocampal slices might likely be associated with synaptic function. This idea need to be addressed in the future.

Surprisingly, we found just a small proportion of dividing cells expressing astroglial marker GFAP with no visible effect of oxygen-glucose deprivation on their proliferation. Our finding remains in agreement with previously published reports showing unremarkable

proliferation of the astroglia in the hippocampus after global ischemia (Kato et al. 2003, Tonchev et al. 2003).

In contrast, after exposure to OGD the vast majority of proliferating BrdU labeled cells expressed antigen ED1- specific for microglia/macrophages. The timing of acceleration of microglial proliferation *in situ* correlates well with the magnitude elevation of cell damage. Significant loss of pyramidal neurons simultaneously with a massive increase of microglia occurred in CA1 at 24–72 h after the insult, whereas in DG was revealed at later time point, between 3–6 days after OGD. The same effect was observed with other microglial marker – Iba1. According to Chechneva and others (2006) OGD-coupled early increase of microglia during the first hours (2–6 in CA1 and 2–16 h in DG) after the insult, a time points well in advance of BrdU incorporation and appearance of neuroblasts, was rather due to microglia migration then proliferation. On the contrary, the presence of the double BrdU/ED1 or BrdU/Iba1 staining detected during the course of our experiment, supports proliferation *in situ.*

Intensive microglia proliferation, pronounced mainly in CA1 area, could play a detrimental as well as beneficial role after ischemic injury (Dirnagl et al. 1999, Schwartz 2003). For example, phagocytosis of injured cells by activated microglia results in the removal of apoptotic cells and debris from damaged cells and subsequent stimulation of reconstruction (Gehrmann et al. 1995, Takahashi et al. 2005). Another likely benefit role of activated microglia may be due to the release of growth factors which can control proliferation, differentiation and cell survival (Yoshimura et al. 2003). Simultaneously, several studies documented the negative impact of microglia proliferation on neurogenesis coupled with the initiation of the neuroinflammatory response induced most probably by the release of cytokines and chemokines (Kreutzberg 1996). Of special interest is observation published by Chechneva and colleagues (2006) that microglia formed clusters of cells that were localized directly in the neurogenic zone of the DG. Thus, the high level of invading microglia may contribute to the observed suppression of neurogenesis in this structure. Beside of all these data it is still not possible to make generalized conclusion concerning the function of OGD-induced amplified microglia proliferation.

Another aim of this work was to study the involvement of metalloproteinases in processes promoted by the deprivation of glucose and oxygen in OHC. As mentioned above, progression of glial cells proliferation after OGD was accompanied by the activation of MMPs during recovery period after the insult. Although this observation does not allow us to state whether there is a casual link between these processes, this prediction is reinforced by already reported *in vivo* studies showing that dynamic evolution of MMPs activity matches the progression of post-ischemic proliferation and differentiation of progenitor cells in dentate gyrus of rodent brains (Lu et al. 2008, Wojcik-Stanaszek et al. 2011). In spite of the activation of MMPs after ischemia is well described, it is not possible to define precisely which of their postulated pleiotropic functions are directly linked to post-ischemic gliogenesis *in vitro.* One likely scenario would involve proteolytic modulation of guidance molecules and/or the remodeling of the ECM during development and homeostasis (Nagase and Woessner 1999). Consistent with this remained emerging *in vitro* and *in vivo* data pointing to the regulatory role of MMPs in neuroblast migration across tissue matrices (Lee et al. 2006, Jablonska and Lukomska 2011). Thus, it is possible that the MMPs, especially MMP-9, are engaged in migration of oligodendrocyte progenitor cells (Uhm et al. 1998, Larsen et al. 2006).

Complementary to the role of MMP9 and MMP12 in OPC migration, they could also promote process extension (Uhm et al. 1998, Oh et al. 1999). This is consistent with the presence of MMPs in developed and matured oligodendrocytes showed in our previous reports (Sypecka et al. 2009, Wojcik-Stanaszek et al. 2011). Although MMPs per se would normally be predicted to promote neural progenitor development, MMP-mediated conversion of several trophic factors, to their biologically active molecules may also produce signals supporting the oligodendrocyte recruitment from neural stem cells and their development (for review see Rivera et al. 2010). The increased gelatinolytic activity in response to OGD was found in all neural cell types. The MMPs-associated activity was noticed in cell cytoplasm, in nuclei and also extracellularly. Thus, the activity of enzymes secreted to the extracellular compartment may result in cleavage of extracellular substrate (Zalewska et al. 2003) and by this may provide unique environment favoring the development of particular progenitors. The localization of MMPs in nuclei is consistent with previous finding (Sbai et al. 2010, Yang et al. 2010, Wojcik-Stanaszek et al. 2011) and most likely

implies that metalloproteinase activity may represent a general mechanism by which neural/progenitor cells adjust their gene expression program involved in cell cycle progression. However, no information is available at present. Nevertheless, regardless on the mechanism of MMPs action it is tempting to speculate about the participation of these enzymes in proliferation of oligodendrocyte precursor cells.

Apart from the hypothesized beneficial role of MMPs in OPC development after ischemic insult, elevated activation of these enzymes in microglial cells, essentially in CA1, may be implicated in pathological functions (for review see Cunningham et al. 2005, Gasche et al. 2006, Rivera et al. 2010). In line with it, the treatment with MMP inhibitors, lead to reduction of inflammation and decreased cell brain damage caused by ischemia *in vivo* and *in vitro* (Machado et al. 2006, Leonardo et al. 2009). Taken together, these data highlight the complexity of MMPs action in exposed to OGD hippocampal slices. Continuing research along this line may provide understanding of ischemia-induced metalloproteinases activation.

CONCLUSION

The findings obtained in the current work show that oxygen-glucose deprivation promotes gliogenesis in organotypic hippocampal slices. Although the functional consequences of OGD-induced activation of metalloproteinases cannot be simply stated, it may comprise an important portion of the response leading to the stimulation of oligodendrocyte progenitors. On the other hand, elevation of MMPs activity may be incorporated into a scenario of postischemic damage of hippocampal cells.

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