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Neurogenesis in gerbil hippocampus following brain ischemia: Focus on the involvement of metalloproteinases

Luiza Wojcik[1], Aleksandra Sawicka[1], Santiago Rivera[2], and Teresa Zalewska[1]*

[1]NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, *Email: terezal@cmdik.pan.pl; [2]UMR 6184 CNRS, Aix-Marseille University, Marseille, France

Accumulating evidence indicates that cerebral ischemia enhances neurogenesis in the adult brain. The mechanisms responsible for stem-cell development are poorly understood. Recent *in vitro* studies indicate the involvement of metalloproteinase (MMPs) in the regulation of proliferation and differentiation of neural progenitor cells. To elucidate if MMPs participate in neurogenesis-associated processes after ischemic insult, we aimed to establish spatial and temporal relationships between neural stem-cell development and the activity of MMPs in the adult brain hippocampus. Our results show that post ischemic acceleration in the proliferation of progenitors in the dentate gyrus is accompanied by increased activity of MMPs. On the contrary, in the damaged CA1 pyramidal layer the neurogenesis seems to be rather elusive. Simultaneously, the activity of MMPs fell below the control level. In conclusion, our results show that the activation of MMPs may, at least in part, contribute to ischemia-induced neurogenesis in the dentate gyrus of the adult brain.

Key words: brain injury, neuronal progenitor, gelatinases, hippocampus

INTRODUCTION

Studies *in vivo* conducted during last 10 years provided substantial evidence that neural stem cells respond to numerous physiological and pathological conditions, including ischemia (Liu et al. 1998, Arvidsson et al. 2002, Kokaia and Lindvall 2003). It was found that ischemia stimulates proliferation and differentiation of neural/stem progenitor cells. Furthermore, newly generated neurons integrate into the already existing neuronal networks. Thus, it has been concluded that expansion of the pool of endogenous progenitors could augment regenerative capacity in response to ischemic insult (Jin et al. 2001, Arvidsson et al. 2002, Nakatomi et al. 2002, Parent et al. 2002, Yamashita et al. 2006).

In the adult brain, neurogenesis is limited to two regions, the gyrus dentatus (DG) of the hippocampus and the olfactory bulb (Altman and Das 1967, Kaplan and Hinds 1977, Kaplan and Bell 1983). Despite growing information concerning progenitor cells, the cellular and molecular mechanisms that regulate post-ischemic appearance of newborn neurons in specific brain structures are poorly understood. In recent years many different molecules (transcription and growth factors, cytokines, adrenal steroids, neurotransmitters) have been described, some of which have been shown to increase after ischemic injury (Cameron and Gould 1994, Cameron et al. 1998, Parent et al. 1997, Liu et al. 1998, Battista et al. 2006, Yan et al. 2006, Arias-Carrion et al. 2007, Catts et al. 2008). In some cases, cell surface and extracellular matrix (ECM) molecules have been shown to provide an environment instructive or permissive to neurogenesis-associated processes, such as proliferation of the cells, migration and differentiation of neuroblasts and glial cells (Luckenbill-Edds 1997, Bovolenta and Fernaud-Espinosa 2000, Bovetti et al. 2007).

ECM remodeling required for many developmental processes, is principally regulated by the family of matrix metalloproteinases (MMPs). MMPs comprise a family of currently 25 distinct vertebrate gene products, 24 of which are found in mammals. MMPs are the secreted or membrane-bound zinc-dependent endopeptidases responsible for the extracellular matrix

Correspondence should be addressed to T. Zalewska
Email: terezal@cmdik.pan.pl

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turnover, the degradation of bioactive proteins, and the shedding of membrane receptors (for review see Dzwonek et al. 2004, Gasche et al. 2006). Although MMPs, in particular gelatinases – MMP2 and MMP9, have been investigated in the context of detrimental roles in brain ischemic injury, recent studies suggest that they are also beneficial for mediating stem-cell proliferation (Mott and Werb 2004, Mannello et al. 2006) and for the differentiation of neural precursor cells in many tissues (Vu and Werb 2000, Bourlier et al. 2005). In concert with the above findings, high expression levels of MMP9 were found in progenitor cells associated with the development of specific structures, such as hypophysis, choroid plexus, and the ganglion cell layer of the retina, as well as in aggregates that would form highly vascular gray matter of the brain (Canete Soler et al. 1995).

It has been hypothesized that MMPs may also be involved in injury repair, favoring the migration of precursor stem cells to injured sites to replenish lost cells (Yong et al. 2001). Cleavage of matrix components by specific metalloproteinase may affect the linkage between the ECM and the signaling pathways that activate transcription factors ultimately determining cell fate.

This prompted us to evaluate the possible involvement of MMPs in neurogenesis. Based on the report indicating that forebrain ischemia in gerbils stimulates appearance of newborn neurons in the damaged CA1 area of hippocampus (Schmidt and Reymann 2002), we used a similar model for the current study. Our attention was primarily focused on the temporal and spatial relationships between the proliferation of neural stem cells and/or differentiation with expression of MMPs at the level of enzymatic activity in brain hippocampus.

METHODS

Reagents

The following antibodies were used: rat polyclonal anti-BrdU (Serotec, 1:200), mouse monoclonal anti-neuronal nuclear antigen (NeuN) (Chemicon, 1:500), mouse monoclonal anti-neurofilament 200 (NF 200) (Sigma, 1:500), rabbit polyclonal anti-GFAP (Dako, 1:1 000). Anti-rat FITC conjugated (Bethyl Lab, 1:1 000), anti-mouse Alexa 546 (Invitrogen, 1:500), anti-rabbit Alexa 546 (Invitrogen, 1:500) respectively, served as the secondary antibodies.

Ischemic model

All experimental treatments were approved by the Local Commission of Ethics of Experiments on Animals.

Male Mongolian gerbils, weighing 50–70 g were used in the experiments. The animals were allowed free access to food and water. Forebrain ischemia was performed as previously described (Domanska-Janik et al. 1999) by 5 minutes bilateral ligation of the common carotid arteries under halothane/N_2O anesthesia in strictly controlled normothermic conditions). Animals subjected to ischemia were allowed to recover up to 2 and 4 weeks after the insult. Sham-operated gerbils served as controls. Each experimental group consisted 6 animals

BrdU labeling

The thymidine analog 5-bromo-2-deoxyuridine (BrdU; Sigma) was administered intraperitoneally (50 mg/kg) twice daily for 3 consecutive days starting 6 days after ischemia.

Tissue preparation and immunohistochemistry

At the estimated survival time after ischemia (2 or 4 weeks) animals were anesthetized and perfused transcardially first with phosphate buffered saline (PBS) followed by a fixative solution (4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4). The brains were removed, post-fixed for 3 hours at 4°C in the same fixative solution. Following post fixation, brains were cryoprotected overnight in 20% sucrose solution (in 0.1M PBS), frozen on dry ice and stored at −70°C. Double-labeled immunofluorescence was performed on free floating 25 µm coronal cryostat sections (three to five per animal) comprising the hippocampal formation of 3 animals per group.

For the BrdU immunostaining, DNA was first denaturated in 2N hydrochloric acid at 37°C for 60 min. After this time tissue sections were incubated in 0.1 M sodium tetraborate (pH 8.5) for 15 min, blocked with 10% normal goat serum in PBS containing 0.25% Triton X-100 for 60 min, and incubated with anti-BrdU overnight at 4°C. After the washing procedure the sections were exposed to secondary anti-rat IgG1 Alexa Fluor 546 antibody for 60 min at room temperature and in the dark.

For monitoring differentiation of BrdU-positive cells, the following primary antibodies were used: mouse NF-200 and mouse NeuN as a neuronal markers and GFAP as an astrocytic marker. After BrdU staining, the brain-tissue sections were incubated with primary antibodies overnight at 4°C. After rinsing in PBS, the sections were exposed by 1 hour at room temperature to secondary antibodies. The adjacent sections served as negative controls. All the procedures for negative controls were processed in the same manner but with the primary antibodies omitted.

To obtain detailed images of the BrdU-positive cells immunoreactive for NF200 and NeuN a confocal laser scanning microscope (Zeiss LSM 510) was used. A helium-neon laser (543 nm) was utilized for excitation of Alexa Fluor 546, while an argon laser (488) was applied for the excitation of FITC.

Cell counting and statistical analysis

To determine the number of BrdU-positive cells, an average of 4 sections per gerbil was used. To avoid double counting we did not analyze adjacent sections. All of the counting was performed under the fluorescence microscope and using 20× objective.

For statistical analysis we used one-way analysis of variance (ANOVA) followed by *post-hoc* Fisher's test. All values are given as mean ± SEM. Differences were considered significant if $P<0.05$.

In situ zymography

In order to localize activity of MMP-2 and MMP-9 within the brain hippocampus in control and ischemic animals (3 gerbils per experimental group) we have conducted *in situ* zymography according to the method described by Rivera and coauthors (2002). Frozen, non-fixed coronal brain sections (25 μm thick) were thawed and incubated for 3 h at 37°C in a humid dark chamber in reaction buffer containing 50 μg/ml of FITC-labeled DQ-gelatin (Molecular Probes, Eugene, OR) that is intramolecularly quenched. Gelatin-FITC cleavage by tissue metalloproteinases (gelatinases) releases peptides whose fluorescence is representative of proteolytic activity.

The sections were rinsed in PBS and fixed in cold 4% paraformaldehyde for 20 min, then mounted in Fluorescent Mounting Medium (Dako) and observed using fluorescence microscopy. To confirm that the proteolytic activity is attributable to MMPs, in each experiment some sections were incubated for 1 h at room temperature in a broad spectrum of the inhibitor of metalloproteinases 1 mM 1,10-O-phenantroline.

In order to identify the cell types expressing gelatinolytic activity, double fluorescent labeling was performed. After zymography, the sections were rinsed in PBS pH 7.4, (3 × 10 min), preincubated in a blocking solution (10% goat serum + 0.5% Triton X-100) followed by overnight incubation at 4°C with either a rabbit polyclonal anti fibrillary acidic protein (GFAP) or anti neuron-specific nuclear protein (NeuN).

RESULTS

Effect of ischemia/reperfusion on proliferation and differentiation of neural progenitors in the brain hippocampus

The proliferation of neural progenitor cells in the dentate gyrus and CA1 sector of the hippocampus was assessed 2 and 4 weeks following forebrain ischemia using BrdU incorporation. To characterize the fate of $BrdU^+$ cells, the sections from sham and ischemic gerbils were double stained with BrdU and NF-200 or NeuN antibodies.

Dentate gyrus

Figures 1 and 2 show that ischemia induced a striking proliferation of the precursor population in the subgranular zone (SGZ) (Fig. 1B–C, E–F), whereas only a few BrdU labeled cells were seen in the control, normoxic animals (Fig. 1A,D). Expansion of BrdU-positive cells was most pronounced at 4 weeks of recovery (Fig. 1C,F). Although a number of BrdU-positive nuclei were associated closely with matured neurons expressing NeuN (Fig. 1D–F), confocal image analysis also revealed cells co-labeled with NF-200, a marker of immature neurons (Fig. 1A–C). Similarly, the double stained cells BrdU/NF200 and BrdU/NeuN were observed in control animals (Fig. 1A,D).

The location of the double stained cells changed during the survival time. At 28 days after ischemia, a number BrdU/NeuN as well as a few BrdU/NF-200 cells were also found throughout the area immediately adjacent to the SGZ, suggesting their short-distance migration.

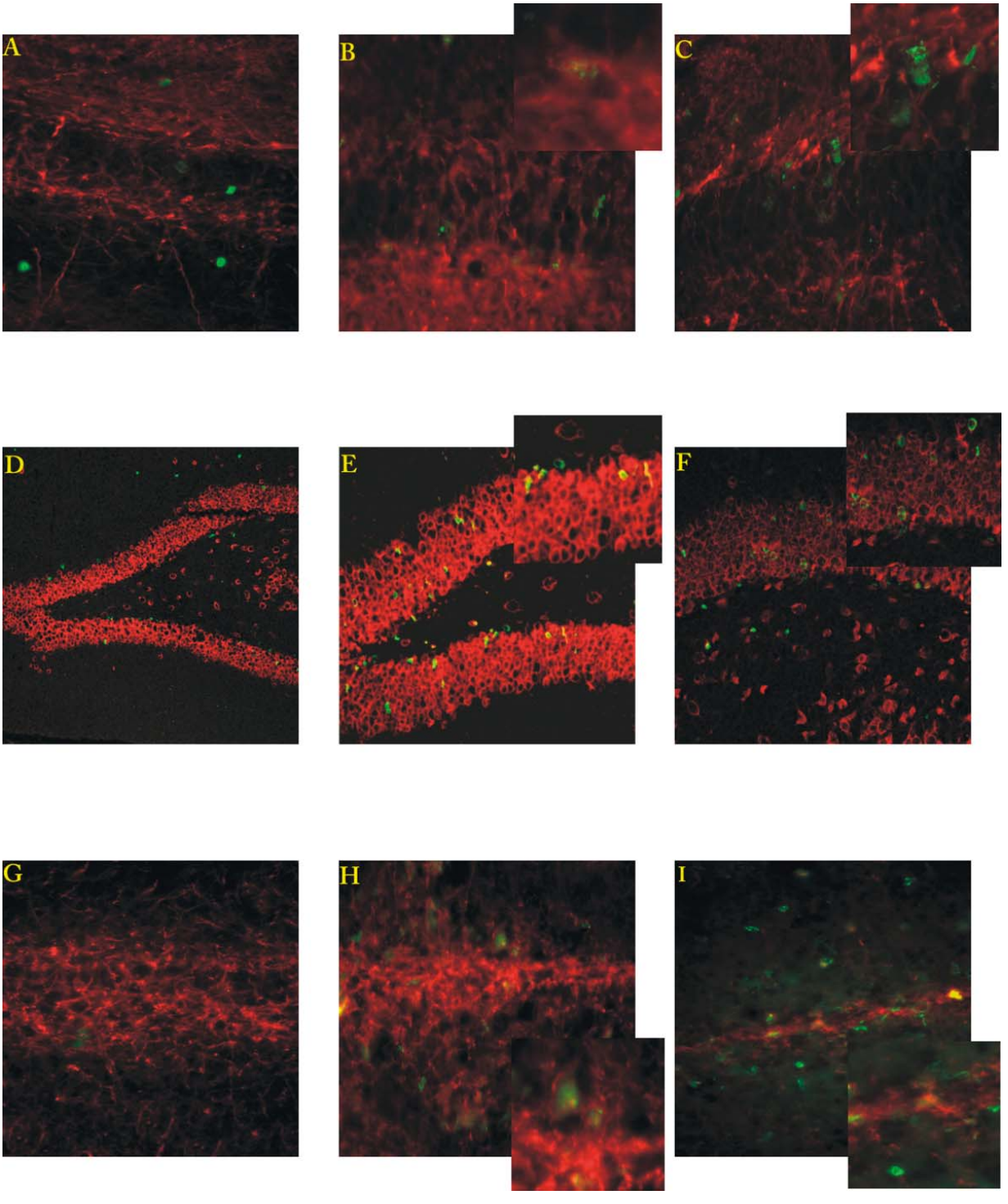

Fig.1. Neurogenesis in the adult gerbil hippocampus after ischemia/reperfusion. Brain sections from control animal (left panels), from an animal 14 days after ischemia (middle panels) and from an animal 28 days following ischemia (right panels) were immunostained for BrdU (green) and neuronal specific markers – NF-200 and NeuN (red) in the dentate gyrus (A–F) and CA1 (G–I). Double-staining for BrdU with NF-200 or NeuN is shown by yellow color. Labeled cells presented on microphotographs A–C, G–I were viewed through a fluorescent microscope (Axioscope 2) with 20× objective. Micrographs D–F were visualized with confocal laser-scanning microscope (Zeiss LSM 510) with 20× (E, F) and 10× objective (D). Photomicrographs are representative of observations made from three animals per time point.

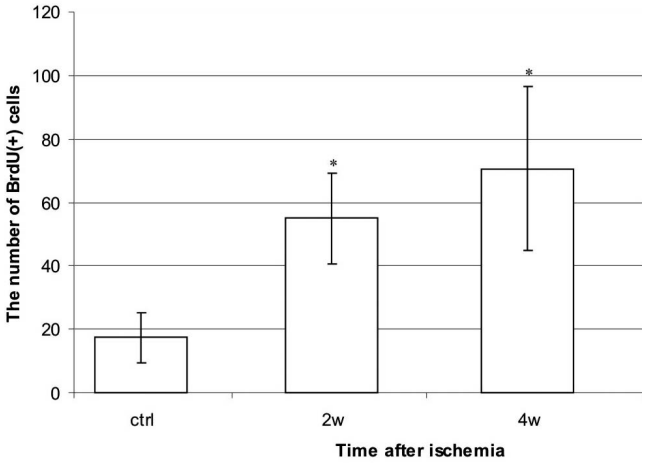

Fig. 2. Rate of cell birth (number of cells labeled with BrdU) 2 and 4 weeks following ischemia in the adult gerbil dentate gyrus compared to controls. The results (mean ± SEM) shown are from three independent animals in each experimental group. *P<0.05.

Cornus Ammoni (CA1)

During the recovery time observed in this study, the number of BrdU positive cells in the ischemia-damaged CA1 sector of the hippocampus was larger than in the controls (Fig. 1H–I vs. G) However, within the destructed pyramidal cell layer, only a few BrdU labeled cells showed co-expression with NF-200 (Fig. 1H,I), but they did not exhibit NeuN antigen.

Effect of ischemia/reperfusion on activity of MMPs in the brain hippocampus

In order to determine whether net gelatinolytic activity parallels post-ischemic development of stem/progenitor cells, we used *in situ* zymography in *ex vivo* brain slices, followed by double staining with anti-NeuN and GFAP antibodies. As shown in Fig. 3 (A,D),

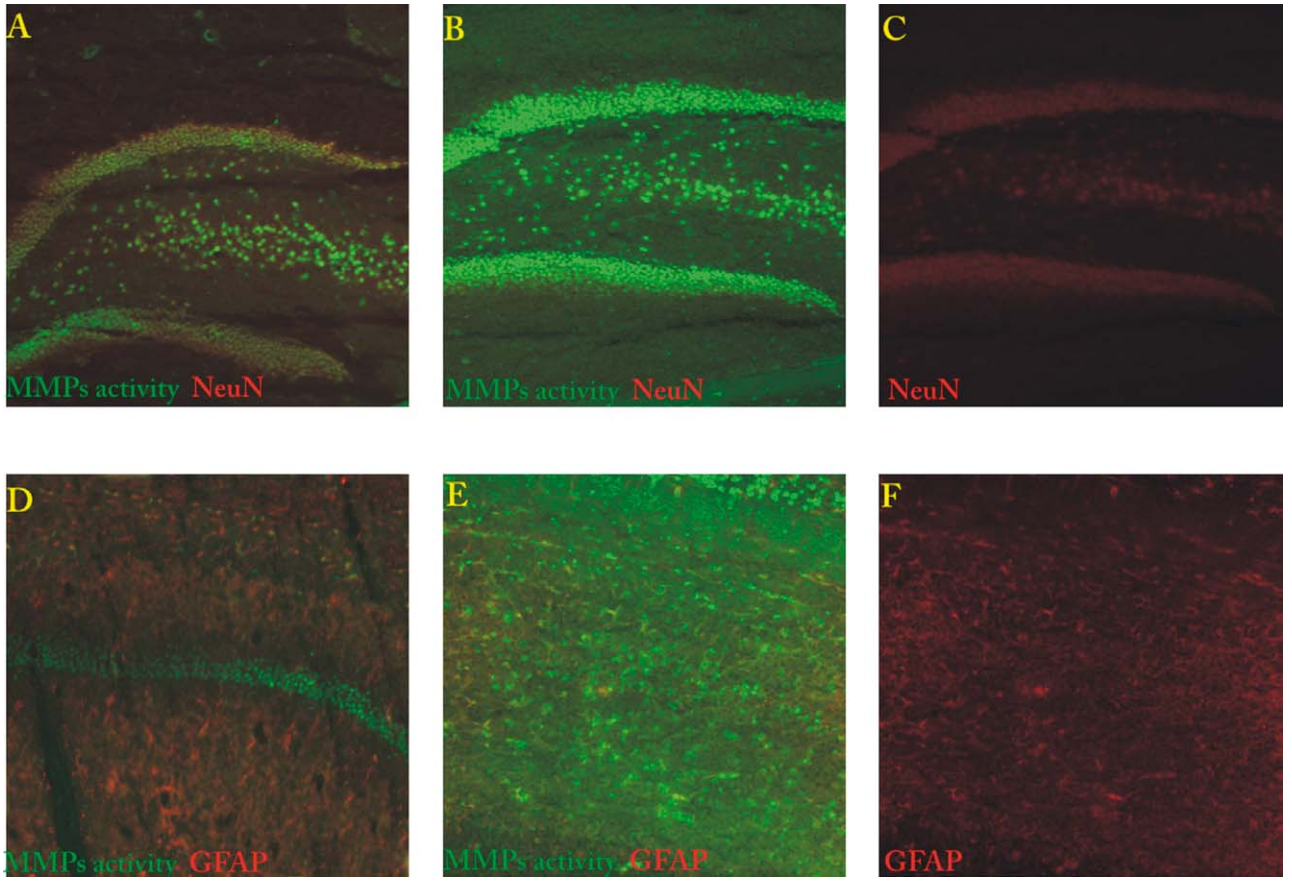

Fig. 3. *In situ* gelatinolytic activity (green, left and middle panels) and NeuN (red, upper right panel) or GFAP (red, lower right panel) stained cells in adult gerbil hippocampus after ischemia/reperfusion. MMPs activity detected as green fluorescence signal in control (left panels) and ischemic brain at 28 days after the insult (middle panels). Micrographs show co-staining of MMPs activity with neuronal marker NeuN in the dentate gyrus (A, B, yellow) and astrocytic marker (GFAP) in CA1 sector (D, E, yellow). Note the association of MMPs activity with neurons in DG and with GFAP labeled cells in CA1.

in the control hippocampi fluorescence signal was principally associated with neurons in the CA1 and DG areas and was virtually absent in glial cells. Forebrain ischemia resulted in a marked enhancement of gelatinases activity in the DG neurons at 14 (not shown) and particularly at 28 days of reperfusion (Fig. 3B). The strong fluorescence appeared to be associated with nuclei, however it was also detected outside the cell bodies. Elevated activity of MMPs in this region coincided spatially and temporarily with an increasing number of BrdU-labeled cells.

At the same reperfusion time following ischemia (4 weeks), a decrease in gelatinase-associated proteolytic activity below the control level was observed in CA1 pyramidal neurons (Fig. 3E). The reduction in fluorescence intensity in this layer contrasted with the clear activation in adjacent regions – strata radiatum and oriens.The activity in both these structures was detected in GFAP immuno-labeled cells and was most pronounced in the extracellular compartment.

DISCUSSION

This report demonstrates that brief forebrain ischemia in gerbils stimulates neurogenesis in the dentate area of the hippocampus, and that this response is accompanied by elevated activity of extracellular metalloproteinases. This may indicate that activation of gelatinases is likely to be involved in neurogenesis-associated processes. Despite that chiefly descriptive character of our results, some important information can be deduced from the temporal and spatial coexistence of both processes. Unlike the relatively well-established pivotal role of MMPs contributing to brain damage after ischemia (Asahi et al. 2000, Rivera et al. 2002, Zalewska et al. 2002, Lee et al. 2004, Magnoni et al. 2004), involvement in post-ischemic endogenous neurogenesis *in vivo* has not been described.

The analysis of the extent of ischemia-induced cellular proliferation aimed in our study shows a marked acceleration of BrdU incorporation in the sub-granular zone of the gyrus dentatus at 14 and 28 days following the insult. It needs to be underlined that BrdU labeled nuclei of both control and ischemic brains appeared as irregularly shaped clusters, which is a characteristic pattern of DG progenitor cells (Gould and Tanapat 1997). Furthermore, most progenitor cells appeared to differentiate into mature neurons, which migrated to the SGZ adjacent area. According to published reports, the stimulation of neural progenitors in DG probably leads to increased production of a new granule cells (Liu et al. 1998, Jin et al. 2001, Yoshimura et al. 2001).

In general, our observations remain in agreement with previously reported data indicating ischemia-stimulated neurogenesis in the DG area. A few unimportant disparities between our results and the results of others can be attributed to the time of differentiation, and may be due to differences in experimental protocols (Liu et al. 1998, Schmidt and Reymann 2002, Sharp et al. 2002).

Despite the extensive neuronal loss in the pyramidal layer of CA1 sector during the initial two weeks after ischemia, we detected a small number of BrdU-labeled nuclei, slightly higher than in the control. We found that some of BrdU positive cells, sparsely distributed within the destructed pyramidal layer, express NF-200 antigen. However, they did not develop into mature neurons, and thus probably undergo apoptosis. Therefore, it can be concluded that endogenous regenerative capacity appears to be very limited and not capable of restoring lost neuronal circuits. However, some surprising results reported by Nakatomi and colleagues (2002) suggest, that new neurons are integrated in the adult brain into the existing brain circuitry. It should be mention that these results have not been reproduced by others.

One of the most interesting findings obtained in the current work is the spatial and temporal relationship between the activation of MMPs and intensive proliferation in the dentate gyrus. Thus, it is tempting to speculate about the participation of these enzymes in neurogenic changes causing by ischemia. Consistent with this notion, there remains emerging *in vitro* data that indicates the involvement of MMPs in neurogenesis and particularly in neuroblast migration across tissue matrices (Tsukatani et al. 2003, Yong 2005, Lee et al. 2006). Direct evidence that gelatinases play a role in neurogenesis-associated processes comes from the recent *in vitro* study performed in our laboratory. We demonstrated that inhibition of MMPs reduced proliferation and differentiation into neurons in cultured human umbilical cord blood-neural stem cell (Dragun et al. 2008). We also found that activation of MMPs may provide a signal for the oligodendroglial lineage commitment (Sypecka et al. 2009, this Issue).

Despite ever-growing information concerning participation of MMPs in neurogenesis, it is not possible to

define precisely which of their known pleiotropic actions are linked to this process. The most probable, and consistent with the established role of MMPs, is proteolytic remodeling of extracellular matrix as well as the modulation of growth factor/cell adhesion molecule functions (Nagase and Woessner 1999). Thus, the activity of enzymes secreted to the extracellular compartment in the DG area may be associated with the formation of a unique environment needed for neural progenitor development and migration (Deryugina et al. 1997, Vaillant et al. 1999, 2003, Bianco et al. 2001, Tsukatani et al. 2003, Zipori 2004, Ayoub et al. 2005, Yong 2005, Lee et al. 2006, Bovetti et al. 2007). Thus, the relocation of progenitor cells from SGZ to the neighboring area observed in the course of our study may be due to the breakdown of ECM barriers that impede cell movement. It is thus hypothesized that MMPs could be also needed to support neurite extension by the newly arrived progenitor cells (Hayashita-Kinoh et al. 2001). Although MMPs per se would normally be predicted to promote neural progenitor development, the proteolytic processing of several trophic factors (Wetzel et al. 2003, Yong 2005) may also produce signals supporting neurogenesis (Calof 1995).

Of particular interest in the current work is the presence of strong activity of geletinases in neuronal nuclei in DG area. Moreover, transient intranuclear expression of MMPs has also been detected in maturating oligodendrocytes (Sypecka et al. 2009, this Issue). Such localization implies that gelatinases may proteolyze transcription factors and thereby cause marked alterations that are involved in the transcription of proneural genes. Confirmation of this scenario will require further investigation. Nevertheless, regardless of the mechanism of MMPs action in DG, the timing and magnitude of their activation, similar to the time frame of accelerated BrdU incorporation, strongly argues for their participation in ischemia-induced neurogenesis.

However, it should be remembered that ischemia-induced neuronal progenitor development may be controlled by cooperative actions of many factors. Multiple stimulating and inhibitory signals are probably involved in regulating neurogenesis-associated processes.

In contrast to DG, the endogenous neurogenesis in the damaged CA1 area seems to be rather elusive, since newborn neurons did not attain maturity. This statement remains in agreement with other published reports (Liu et al. 1998, Sharp et al. 2002), suggesting that generation of CA1 pyramidal neurons should be a rare event. Supposedly, the ischemia-induced toxicity of the environment at the site of injury, as well as a glial scar, may limit the regeneration process (Fawcett and Asher 1999, Ribbota et al. 2004, Markiewicz and Lukomska 2006). At the time points investigated in this study, the gelatinolytic activity in the degenerated pyramidal layer dropped below the control level, probably due to the massive neuronal loss caused by forebrain ischemia. Although our current findings do not allow us to state whether there is a casual link between attenuation of MMPs activity and the failure of cells to mature, this prediction may be reinforced by the temporal and spatial coexistence of the above processes. It may be speculated that the reduced activity of metalloproteinases at injury site is not able to sufficiently degrade the inhibitory molecules arising by ischemic insult and thereby unable to make the environment more permissive (Muir et al. 1998).

Contrary to pyramidal cells layer, we found significant enhancement of gelatinolytic activity in the stratum radiatum and stratum oriens. According to our previous report, ischemia induced changes are located in the dendritic region of CA1 pyramidal neurons of stratum radiatum and then precipitates to stratum oriens (Domanska-Janik et al. 2001, Ziemka-Nalecz et al. 2003). In these regions, stress response of post injured tissue and late formation of glial scar seems to be associated with the appearance of activated astrocytes. High activity of MMPs detected in both structures shows clear preference to astrocytes and probably plays a role during attempts at delayed post ischemic tissue remodeling and delayed endogenous repair processes (Dusart et al. 1991).

CONCLUSIONS

Our results presented here show for the first time that MMPs may, at least in part, contribute to neurogenesis observed after ischemic insult in the adult DG along with previously described molecules, including transcription and growth factors, cytokines, adrenal steroids and neurotransmitters.

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