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HIGH RESOLUTION *IN SITU* ZYMOGRAPHY REVEALS MATRIX METALLOPROTEINASE ACTIVITY AT GLUTAMATERGIC SYNAPSES

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Abstract—Synaptic plasticity involves remodeling of extracellular matrix. This is mediated, in part, by enzymes of the matrix metalloproteinase (MMP) family, in particular by gelatinase MMP-9. Accordingly, there is a need of developing methods to visualize gelatinolytic activity at the level of individual synapses, especially in the context of neurotransmitters receptors. Here we present a high-resolution fluorescent *in situ* zymography (ISZ), performed in thin sections of the alcohol-fixed and polyester wax-embedded brain tissue of the rat (*Rattus norvegicus*), which is superior to the current ISZ protocols. The method allows visualization of structural details up to the resolution-limit of light microscopy, in conjunction with immunofluorescent labeling. We used this technique to visualize and quantify gelatinolytic activity at the synapses in control and seizure-affected rat brain. In particular, we demonstrated, for the first time, frequent colocalization of gelatinase(s) with synaptic N-methyl-D-aspartic acid (NMDA)- and AMPA-type glutamate receptors. We believe that our method represents a valuable tool to study extracellular proteolytic processes at the synapses, it could be used, as well, to investigate proteinase involvement in a range of physiological and pathological phenomena in the nervous system. © 2009 Published by Elsevier Ltd on behalf of IBRO.

Extracellular proteolytic enzymes are emerging new players among mechanisms of synaptic plasticity. Such a role has been most firmly established for the family of serine proteinases, including, among others, plasminogen activator (tPA)/plasmin system. The evidence includes involvement in long-term potentiation (LTP), some forms of learning and/or epileptogenesis (reviewed by Shiosaka (2004)). Yet another class of extracellular proteinases that have been recently implicated in synaptic plasticity are matrix metalloproteinases (MMPs), a large family of structurally related, cell-membrane-bound or extracellularly-released enzymes, that are considered to be the major effectors of extracellular matrix remodeling throughout the body (Sternlicht and Werb, 2001). In the adult brain, MMPs have been repeatedly implicated in various pathological conditions, including acute and chronic neurodegenerative processes (e.g. ischemia, trauma and Alzheimer's disease), inflammation, and cancer (Yong, 2005). Among several MMPs that are expressed in brain, MMP-9 and MMP-2 have been the most studied neuronal and glial MMPs, respectively (Dzwonek et al., 2004). They are collectively referred to as gelatinases for their ability to cleave gelatin, a denatured form of collagen I, *in vitro* (Woessner and Nagase, 2000). MMP-9 is required for late-phase LTP in hippocampus (Nagy et al., 2006), prefrontal cortex (Okulski et al., 2007) and amygdala (Balcerzyk and Kaczmarek, unpublished observations), as well as for spatial and emotional learning (Meighan et al., 2006; Nagy et al., 2006). It is also involved in epileptogenesis (Wilczynski et al., 2008). Importantly, MMP-9 appears to exert its action directly at the level of dendritic spines (Konopacki et al., 2007; Tian et al., 2007; Wilczynski et al., 2008), perhaps by cleaving synapse-associated molecules such as β-dystroglycan (Michaluk et al., 2007) and/or intracellular adhesion molecule 5 (ICAM-5) (Tian et al., 2007). Although much has been learned already, further progress in the field requires developing methods of visualization of MMP activity at the level of individual synapses, in the context of neurotransmitters receptors, ion channels and other key players in synaptic plasticity.

Fluorescent *in situ* zymography is a method of choice for high-resolution visualization of MMP activity. It utilizes, a special fluorescein-gelatin conjugate: dye-quenched (DQ-) gelatin, in which the fluorophore molecules are so tightly packed that they quench one another (reviewed in (Frederiks and Mook, 2004)). It is believed that upon cleavage of DQ-gelatin, the quenching is relieved, and the product becomes fluorescent at the site of gelatinase action (Frederiks and Mook, 2004). Typically, the technique is applied to unfixed frozen sections of the brain, hence, due to severe compromise of the fine-structural details, at best, only moderate-resolution approach is feasible (Oh et al., 1999; Gu et al., 2002; Szklarczyk et al., 2002; Jourquin et al., 2003; Lee et al., 2004). Here we present our attempt to

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Abbreviations: CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DQ, dye-quenched; GFAP, glial fibrillary acidic protein; GluR2/3, glutamate receptor subunits 2 and 3; ICAM-5, intracellular adhesion molecule 5; LTP, long-term potentiation; MMP, matrix metalloproteinase; NDS, normal donkey serum; NMDA, N-methyl-D-aspartic acid; PBS, phosphate buffered saline; PFA, paraformaldehyde; tPA, plasminogen activator.

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improve the existing *in situ* zymography protocols to study at the limit of light microscopy resolution the colocalization of gelatinase activity with synaptic markers, including N-methyl-D-aspartic acid (NMDA) and AMPA types of glutamate receptors, in the rat brain.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed in 12 adult male Wistar rats according to the rules established by the Ethical Committee on Animal Research of the Nencki Institute, based on the Polish Act on Animal Welfare and other national laws that are in agreement with European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123). All efforts were made to minimize the number of animals used and their suffering. Kainate (Ocean Produce, Shelburne, Canada) was injected into the peritoneal cavity at the dose of 10 mg/kg, and the animals were observed for up to 6 h, as described (Szklarczyk et al., 2002). At specified time points after injections, the animals were killed by decapitation, and their brains were immediately removed and fixed (see below).

Tissue processing

Isolated brain fragments (coronal sections of brain tissue up to 5 mm in thickness) were immersed in alcoholic fixative composed of one volume of methanol and three volumes of 95% ethanol, for 6 h, at 4 °C, and subsequently moved to the freezer at −20 °C, to be stored therein overnight. The brains were then washed in 99.8% ethanol (4 °C), and carried through several mixtures of ethanol and polyester wax (Science Services, Munchen, Germany) with increasing concentration of the latter, and with progressive raising the temperature up to 42 °C (in pure wax). Finally the specimens were embedded in 100% polyester wax. Blocks were cut into 6 μm-thick sections using a rotary microtome equipped with the running water path (RMC, Boeckeler, Germany); the sections were mounted on silane-coated slides and kept at 4 °C until assayed (up to several weeks). The remaining blocks were stored at −20 °C for several months without any adverse effect on the gelatinolytic activity.

For Western blotting, isolated hippocampi were snap frozen on dry ice and then stored at −80 °C until needed.

In situ zymography

The sections were dewaxed in absolute ethanol (37 °C, two times for 5 min and 10 min, respectively). Afterward, the alcohol was removed and slices were hydrated with phosphate buffered saline (PBS), pH 7.4, and *in situ* zymography was performed as follows: the specimens were first pre-incubated in water at 37 °C for 40 min, and then overlaid with a fluorogenic substrate DQ gelatin (Invitrogen/Molecular Probes, Eugene, OR, USA) diluted 1:100 in the buffer supplied by the manufacturer, for 40 min at 37 °C. Then, they were washed with PBS, and fixed in 4% PFA, at room temperature (RT), for 15 min. Next the slides were either mounted directly in Vectashield (Vector, Burlingame, CA, USA) or subjected to immunofluorescent procedure. The specificity of the reactions was examined using chemical MMP inhibitors: a) phenanthroline (Molecular Probes) at the concentrations ranging from 1.4–140 mM, b) GM6001 (Millipore, Billerica, MA, USA) at 50 μM to 5 mM concentrations, and c) "MMP-9 inhibitor I" (Calbiochem/Merck Biosciences, San Diego, CA, USA) at 25–500 μM concentrations. To check for unspecific effects, the reactions were also performed in the presence of solvents used to prepare stock solutions (DMSO in case of GM6001, ethanol in case of phenanthroline and "MMP-9 inhibitor I") at appropriate concentrations. At least three independent stainings were performed per each animal.

Immunofluorescence

The sections were (1) blocked for 2 h in 5% normal donkey serum (NDS, Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, UK) in PBS, (2) incubated overnight at 4 °C simultaneously with two primary antibodies diluted with NDS, (3) sequentially incubated with two appropriate, fluorophore-conjugated species-specific secondary antibodies (each for 1 h), (4) washed with PBS and with distilled water, (5) allowed to dry and (6) coverslipped using Vectashield. The following combinations of primary, and secondary (all from Invitrogen), antibodies were applied: (1) rabbit polyclonal anti-NR1 (1:100, Sigma, St. Louis, MO, USA) and mouse monoclonal anti-microtubule-associated protein-2 (MAP2, 1:200, Sigma) followed by donkey anti-rabbit conjugated with Alexa Fluor 555 and donkey anti-mouse conjugated with Alexa Fluor 647 (1:200); (2) rabbit polyclonal anti-GluR 2/3 (1:100, Millipore) and monoclonal anti-MAP-2 (1:200) followed by donkey anti-rabbit conjugated with Alexa Fluor 555 (1:100), and donkey anti-mouse conjugated with Alexa Fluor 647 (1:200), (3) rabbit polyclonal anti-neurofilament 200 (1:100, Sigma) and mouse monoclonal anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (1:200, Sigma) followed by donkey anti-mouse conjugated with Alexa Fluor 555 (1:100) and donkey anti-rabbit conjugated with Alexa Fluor 647 (1:100), (4) rabbit polyclonal anti-gephyrin (1:1000, Abcam) and mouse monoclonal anti-MAP-2 (1:200), followed by donkey anti-rabbit conjugated with Alexa Fluor 555 (1:100), and donkey anti-mouse conjugated with Alexa Fluor 647 (1:200), (5) guinea-pig anti-Drebrin (1:100, Fitzgerald Industries International Inc., Concord, MA, USA) and mouse monoclonal anti-MAP-2 (1:200) followed by donkey anti-guinea-pig conjugated with Alexa Fluor 555 (1:100) and donkey anti-mouse conjugated with Alexa Fluor 647 (1:100), (6) rabbit anti-MMP-9 N-terminal antibody (Millipore, formerly Chemicon, cat. no. AB805) diluted 1:200, followed by donkey anti-rabbit conjugated with Alexa Fluor 555 (1:100).

For fluorescent staining of glial cells, the slides were blocked in PBS with 5% NDS, incubated overnight, at 4 °C, with mouse monoclonal antibody anti–glial fibrillary acidic protein (GFAP) conjugated to Cy3 (1:200, Sigma), and biotin-labeled isolectin B_4 from *Bandeiraea simplicifolia* (1:50, Sigma, L-2140) diluted in PBS containing 0.05 mM $CaCl_2$ and 0.05 mM $MgCl_2$, followed by streptavidin conjugated to AlexaFluor 647 (1:200), washed briefly with distilled water, allowed to dry and coverslipped using Vectashield.

Western blotting

Frozen hippocampi were sonicated (Branson sonifier S250D) in a buffer containing protease inhibitors. The extracts were subjected to SDS-PAGE under reducing conditions, and separated proteins were transferred onto PVDF membrane (Mini Trans-Blot transfer cell from Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked with 3% non-fat milk in PBS and incubated overnight with the same anti-MMP-9 antibody as used for microscopy, diluted 1:1000. Then the membrane was incubated with secondary peroxidase conjugated anti-rabbit antibody from Amersham (Amersham Biosciences, Piscataway, NJ, USA), and subjected to chemiluminescent detection with "ECL Plus" kit (Amersham) followed by exposure to film (Foton XR-1N; Fotochemische Werke GmbH, Berlin, Germany).

Image processing

For final inspection, the images were processed using Corel Package. To restore higher resolution, confocal images were deconvolved using Huygens Professional software (Scientific Volume Imaging,