

# Expression of the type 1 metalloproteinase in the rat hippocampus after the intracerebroventricular injection of $\beta$ -amyloid peptide (25–35)

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The expression of matrix metalloproteinase of the first type was studied in frontal sections of the adult rat brain one month after a single intracerebroventricular injection of  $\beta$ -amyloid peptide (25–35), which is known to be a well-known model of the development of Alzheimer's disease. Brain sections were stained immunocytochemically to detect MMP-1 expression, and histologically to reveal the state of hippocampal neurons. Administration of  $\beta$ -amyloid peptide induced a significant degeneration of cells in the dorsal hippocampus. This was demonstrated by a significant decrease in the total number of cells and by the appearance of acidophilic neurons of altered (often triangular) shape. Altered cells were most often found in the hippocampal field CA3, and in a smaller quantity in the CA1 field. MMP-1-like immunoreactivity was found in the same hippocampal areas, the staining being restricted to the cells of altered shape (staining of somata and primary neurites). The data suggest possible involvement of the type 1 metalloproteinase in the development of Alzheimer's disease.

Key words: metalloproteinase, Alzheimer's disease,  $\beta$ -amyloid peptide, hippocampus

One of the intensively-studied neurodegenerative diseases is Alzheimer's disease (AD). The toxicity of  $\beta$ -amyloid peptide (A $\beta$ ) is presumed to be the main cause of this type of neurodegeneration (Selkoe 2001). AD may result from the accumulation of  $\beta$ -amyloid peptide in the brain (Miners et al. 2008, Rosenberg 2009). It has been postulated that the development of amyloid plaques in Alzheimer's disease may result from an imbalance between the generation and clearance of A $\beta$ .  $\beta$ -amyloid-degrading endopeptidases are thought to protect against Alzheimer's disease (Huang et al. 2008). A $\beta$  clearance involves several A $\beta$ -degrading enzymes, including insulin-degrading enzyme, neprilysin, endothelin-converting enzyme, plasmin, and matrix metalloproteinase-9 (Asashina et al. 2001, Mueller-Steiner et al. 2006). Intraventricular administration of  $\beta$ -amyloid peptide (25–35) is a well-known model of development of AD-like pathology, and it has been demonstrated that A $\beta$  selectively impairs the neu-

rons of the hippocampus causing the impairment of space memory and moderate degeneration of the hippocampal neurons in a month after the administration (Stepanichev et al. 2005, 2006).

A family of matrix metalloproteinases (MMPs) includes more than twenty members (Kaczmarek et al. 2002). They are calcium-requiring, zinc-containing endopeptidases that constitute a major component of the enzyme cascade responsible for degradation of extracellular matrix proteins such as collagen, proteoglycan and laminin (Deb and Gottschall 1996), while the substrate specificity for any one of them is still questionable (Leppert et al. 2001). Most MMPs are not constitutively expressed (Yong et al. 2001, Kaczmarek et al. 2002). MMP-9 was detected in the hippocampus under normal conditions (Szklarczyk et al. 2002), and an abundant expression of MMP-9, MMP-24 and MMP-3 was observed in the adult cerebellum (Vaillant et al. 1999, Sekine-Aizawa et al. 2001). MMPs were shown to be activated by kainic acid-induced seizure activity (Zhang et al. 2000, Jourquin et al. 2003). Their exact role in the development of pathologies is still questionable (Rosenberg 2009).

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The role of MMP-1 is poorly studied both in the normal and pathological brain. At basal conditions, the MMP-1 level is non-detectable or low (Pagenstecher et al. 1997, Leake et al. 2000). In rabbits, the presence of MMP-1 in neurons was detected immunocytochemically in the granular layer of the olfactory lobe (Del Bigio and Jacque 1995). The level of MMP-1 was enhanced in glioblastomas (Stojic et al. 2008), but was unchanged in Parkinson's disease patients (Lorenzl et al. 2002). Nevertheless, in a mouse model of Parkinson's disease, increased levels of mRNA encoding MMP-1 were detected (Hamill et al. 2007), thus suggesting that MMP-1 participates in the development of this disease. In pathological cases, such as multiple sclerosis, MMP-1 was detected immunocytochemically in brain microglia and astrocytes (Deb and Gottschall 1996, Yong et al. 1998, Leppert et al. 2001, Hamill et al. 2005), but not in neurons. In our recent work (Ierusalimsky and Balaban 2013), we detected anti-MMP-1 immunoreactive staining in neurons of several rat brain areas, both in normal conditions and after the 4 h action of kainate (10 mg/kg). In the latter case, the number of brain structures containing the MMP-1 immunoreactive neurons increased. Only one study points out that MMP-1 is possibly involved in Alzheimer's disease: the MMP-1 level was significantly elevated in cortex of AD patients (Leake et al. 2000). We addressed the question of whether MMP-1 expression is changed in the rat hippocampus in the A $\beta$  (25–35) model of Alzheimer's disease.

Adult male Wistar rats (250–300 g) were housed under a 12 h light-darkness cycle at 22–24°C. Experiments were performed in accordance with international regulations for studies involving laboratory animals using a protocol approved by the Ethics Commission of the Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences. Each experimental rat received a single intracerebroventricular injection of  $\beta$ -amyloid peptide (25–35, Bachem), preaggregated as described by Maurice and coauthors (1996) at a total dose of 15 nmoles (7.5 nmoles dissolved in 5  $\mu$ l of water in each ventricle). Control animals received the same volume of distilled water.

Rats were anaesthetized with chloral hydrate and sacrificed at one month after the injection. The brains were rapidly (2 minutes) removed and fixed in the modified Telluescky-Lillie fixative (Lillie 1965). The middle part of the brain (containing hippocampus) was

dissected with a razor blade, and after alcohol-chloroform series was mounted in paraplast and sectioned at a thickness of 10  $\mu$ m in a frontal plane. Each tenth section was mounted on a glass slide. The state of the hippocampal neurons was checked in parallel sections by means of histological staining. We used a routine cresyl violet staining, and two methods for detecting the acidophilic neurons, modified by Victorov and colleagues (2000): vanadium acid fuchsin-toluidine blue staining, and hematoxylin-vanadium acid fuchsin staining. Five control and five A $\beta$ -injected animals were used.

The total number of cells as well as the number of damaged cells was counted in cresyl violet stained sections in four selected hippocampal areas: dorsal and dorso-lateral areas of the CA1 field, dorsal area of the CA3 field, upper and lower parts of the dentate gyrus (see Fig. 1E). The number of MMP-1-immunoreactive cells was counted in the same areas. We counted the cells in 500  $\mu$ m-long stripes of cells in the corresponding areas. Damaged cells were detected due to their acidophilic staining and altered shape (often a triangle). The number of neurons was calculated as an arithmetic mean number of neurons in the left and right hemispheres, and as the arithmetic mean number of data obtained in four sections, corresponding to the lists 37, 39, 41, and 42 of the Rat Brain Atlas (Paxinos and Watson 1998). Thus, for each animal we received one data set for any studied hippocampal area. The data were counted using a correction factor introduced by Abercrombie (1946), and were analyzed using Student's *t*-test. Counts were made using images captured with an AxioCam HRC digital camera (Zeiss, Germany) attached to an Axioskop-2 FS plus microscope (Zeiss, Germany).

Prior to incubation with the primary antibody solution, sections were washed for 2 h in a blocking solution. The blocking solution contained 0.5% Triton X-100, 0.01% sodium azide, 5% normal goat serum (Sigma), and 1% BSA (Sigma) in PBS. The staining procedure was carried out at 4°C using blocking solution for all the washes and antibody dilutions: primary antibody for 24 h; wash for 2 h; secondary antibody for 24–36 h; wash for 2 h. Primary antibody was rabbit anti human MMP-1 (N-terminal) antibody (ABD Serotec) used at 1:80 dilution. The cross-reactivity of this antibody with rat is known from the supplier's data. Supplier recommends the heat retrieval of antigen. Our pilot experiments have shown the same

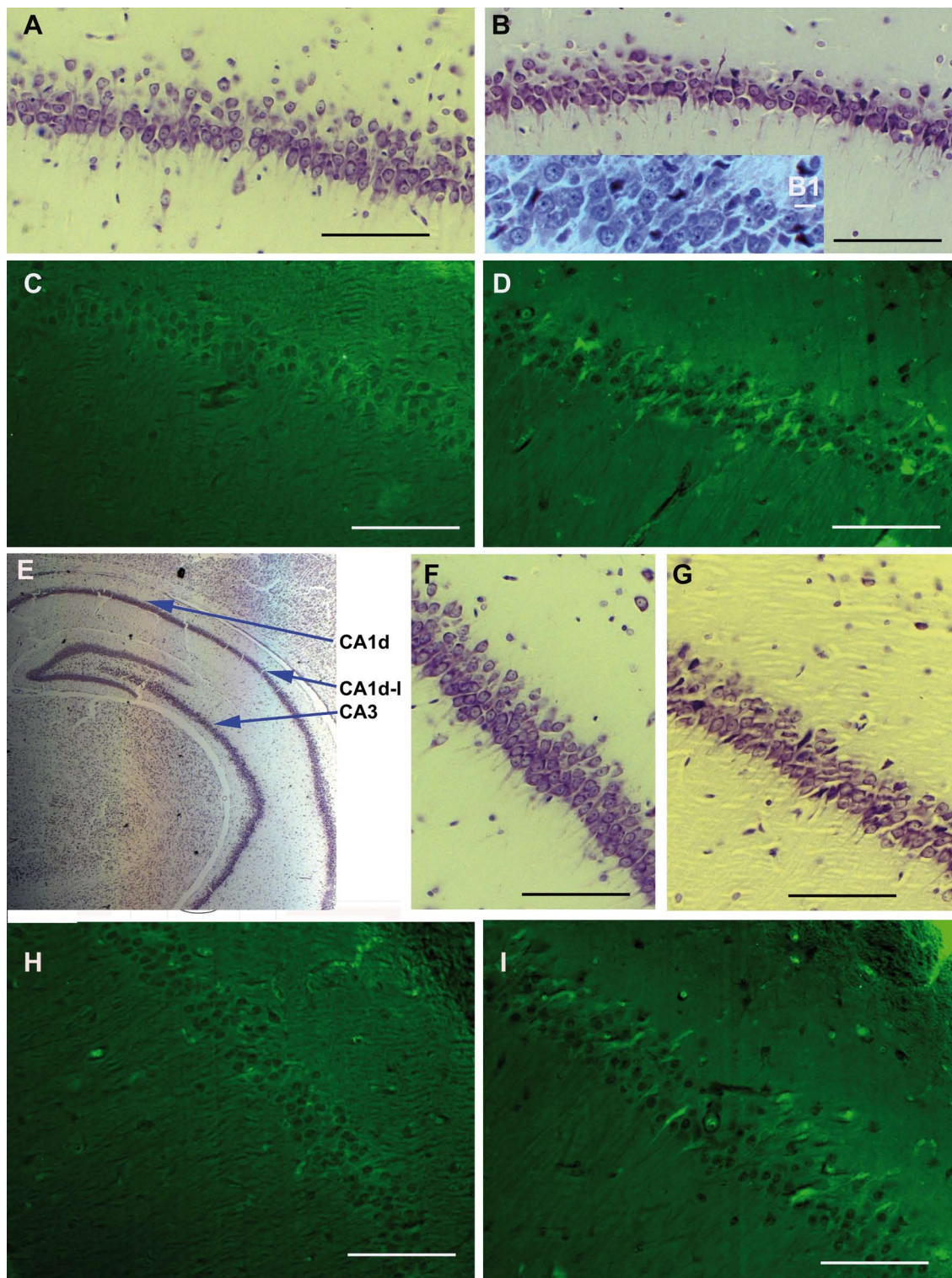


Fig. 1. Morphological changes caused by A $\beta$  (25–35) in the CA1 area of hippocampus. (A–D) dorsal part of the CA1 area. (A, B) cresyl violet staining of normal (A), and experimental (B) brain. (B1) hematoxylin staining of the experimental brain. (C, D) MMP-1-immunoreactivity in normal (C), and experimental (D) brain. (E) low-magnification frontal brain section with arrows pointing to the areas of interest (CA1 d – dorsal part of area CA1, CA1d-l – dorso-lateral part of area CA1). (F–I) dorso-lateral part of the CA1 area. (F, G) cresyl violet staining of normal (F), and experimental (G) brain. (H, I) MMP-1-immunoreactivity in normal (H), and experimental (I) brain. Scale bars are: 100  $\mu$ m (A–D, F–I), 10  $\mu$ m (B1)

results with or without a retrieval step. So, in the next experiments we omitted the retrieval stage. In our previous work with the same antibody (Ierusalimsky and Balaban 2013), we have detected in Western blot experiments only one stripe (about 52 kDa) corresponding to the pro-form of the MMP-1, both in the normal brains and in the brains of kainate-injected rats. Secondary antibody was Alexa-488-conjugated goat anti-rabbit IgG (1:100, Molecular Probes). In the control experiments, the primary antiserum was omitted. No staining was observed in these series. Finally, the sections were embedded in Aqua Poly/Mount (Polysciences, Inc) or in SlowFade Gold antifade reagent with DAPI (Invitrogen).

Injection of A $\beta$  (25–35) led to a significant decrease in the total number of cells in both CA1 (dorsal, and dorso-lateral) areas, and in the dorsal CA3 area (Fig. 2A). The total number of cells in the 500  $\mu$ m band constituted (in control and experimental animals, correspondingly)  $72 \pm 3$  and  $61 \pm 3$  in the dorsal CA1 area ( $P < 0.05$ ),  $79 \pm 4$  and  $58 \pm 7$  in the dorso-lateral CA1 area ( $P < 0.05$ ),  $55 \pm 3$  and  $43 \pm 4$  in the dorsal CA3 area ( $P < 0.05$ ). No statistically significant difference was detected in the dentate gyrus. Thus, the total number of cells decreased mainly in the dorso-lateral CA1 area and in the dorsal CA3 area.

The total population of damaged cells was found to be  $0.9 \pm 0.5\%$  in the CA1 dorsal area, and  $1.0 \pm 0.5\%$  in the CA1 dorso-lateral area in control animals, while in the experimental animals (Fig. 2B) these values were, correspondingly,  $4.9 \pm 0.9\%$  ( $P < 0.05$ ) and  $7.8 \pm 1.1\%$  ( $P < 0.001$ ). The data are illustrated in Figure 1A, B, B1, F, G. In the dorsal part of the CA3 area, the damaged cells constituted  $3.7 \pm 0.7\%$  of total population in the control animals, and  $12.7 \pm 1.9\%$  in the experimental animals ( $P < 0.001$ , see Fig. 2C, D, D1, D2). No statistically significant difference between control and experimental animals was detected in the number of damaged cells in the dentate gyrus (Fig. 2B). Thus, the most evident changes were found in the dorso-lateral part of the CA1 area and in the dorsal CA3 area.

No MMP-1-like immunoreactive elements were detected in control animals in all studied hippocampal areas (Fig. 1C, H; Fig. 2E). In the experimental animals, we found MMP-1-like immunoreactivity (Fig. 1D, I; Fig. 2F) in both dorsal and dorso-lateral CA1 regions ( $3.4 \pm 0.5\%$ , and  $4.2 \pm 0.7\%$ , correspondingly), and in the dorsal CA3 region ( $6.2 \pm 1.3\%$ ). The immu-

noreactivity was detected mostly in the cells of irregular form (damaged cells) – both in the cell bodies and in primary neurites (Fig. 1D, I; Fig. 2F, I). A few cells of normal shape were immunoreactive. In some other cases, MMP-1-like immunoreactivity was found either in the cell bodies of irregular shape (Fig. 2G), or in the distorted (spindle-shaped) neurites (Fig. 2H). No MMP-1-like immunoreactive elements were detected in the gyrus dentata of the experimental animals.

The experiments in animals with intracerebroventricular administration of synthetic A $\beta$  have shown the pathological effects of A $\beta$  on learning and memory processes (Hsiao et al. 1996, Westerman et al. 2002). The (25–35) fragment of A $\beta$  is the functional domain of A $\beta$ , responsible for its neurotoxic properties (Yankner et al. 1990, Pike et al. 1993). Intracerebroventricular administration of A $\beta$  (25–35) impaired different forms of spatial memory (Maurice et al. 1996, Delobette et al. 1997, Yamaguchi and Kawashima 2001, Stepanichev et al. 2005, 2006). It also impaired working memory in rats without any significant effect on the retention of responses (Stepanichev et al. 2005). A $\beta$  injections were accompanied by the death of nerve cells in the brain areas responsible for learning and memory processes (neocortex, fields CA1 and CA3 of hippocampus, and basal nuclei), and by the activation of astrocytes and microglial cell proliferation (Giovannelli et al. 1995, Maurice et al. 1996, Stepanichev et al. 2006).

Morphologically, neurofibrillary tangles and senile plaques composed of extracellular amyloid deposits are the two defining pathological hallmarks of Alzheimer's disease (Helbecque et al. 2003). Though no such signs are detectable in the A $\beta$  (25–35) model of AD, the impairments of memory and learning caused by the A $\beta$  (25–35) injection are similar to the Alzheimer's disease case (Stepanichev et al. 2005, 2006).

We detected the same type of neurodegeneration in the CA1 and CA3 fields of hippocampus (a significant decrease of total number of cells and the appearance of eosinophilic cells with altered shape) as was reported previously (Stepanichev et al. 2006). In their study the damaged cells constituted 5% of the population in the CA3 area (Stepanichev et al. 2006) while we observed more noticeable changes. Nevertheless, we detected the same type of effect (damage of neurons mostly in the CA1 and CA3 dorsal areas) as was reported by Stepanichev and others (2006). In our experiments it



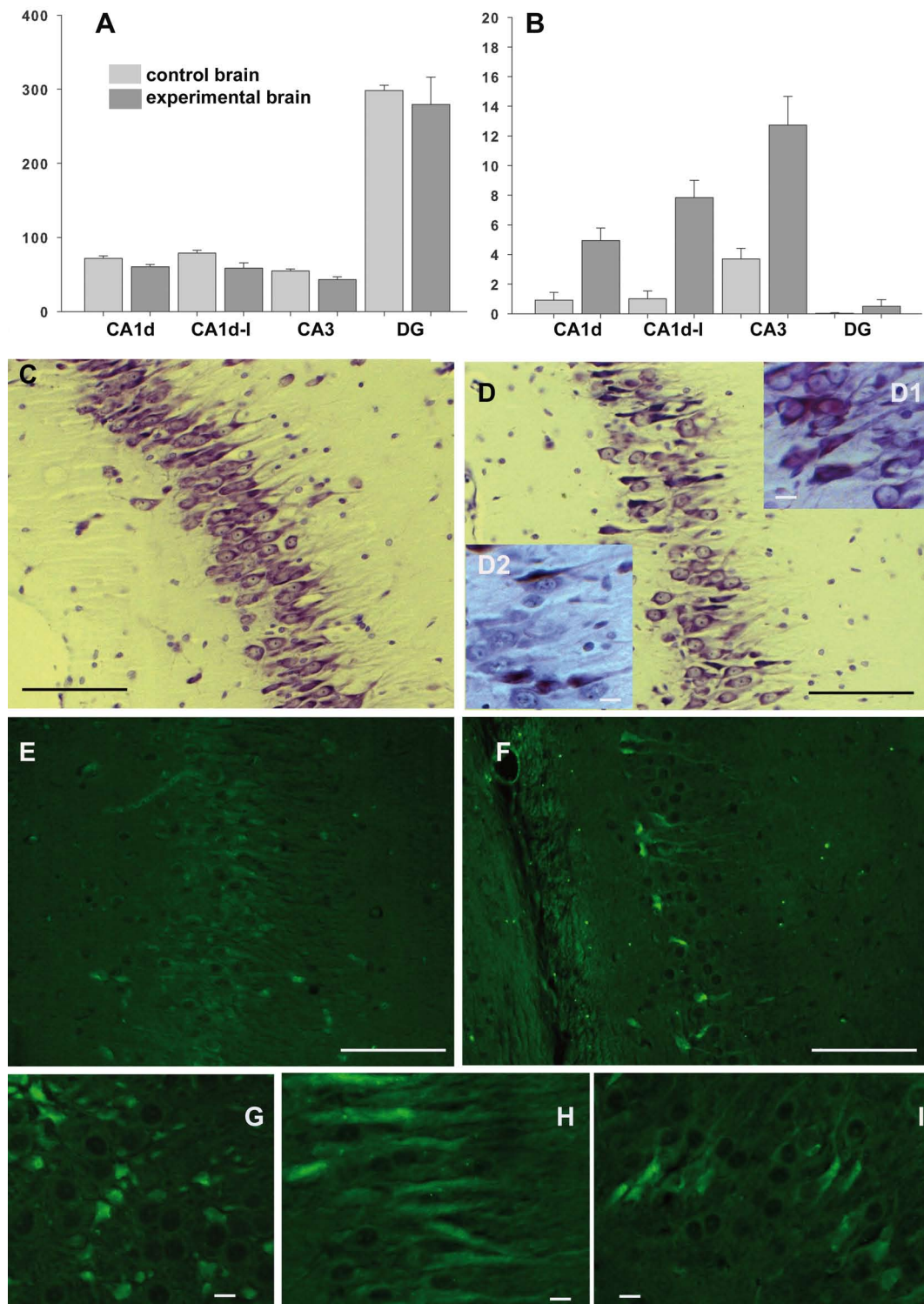


Fig. 2. (A) Total number of cells in the different hippocampal areas per 500  $\mu\text{m}$  of its length. (B) proportion of damaged cells in the different hippocampal areas. (C–I) Morphological changes caused by  $\text{A}\beta$  (25–35) in the CA3 area of hippocampus. (C, D) cresyl violet staining of normal (C), and experimental (D) brain. (D1) fuchsin/toluidine blue staining, (D2) hematoxylin/fuchsin staining. (E, F) MMP-1-immunoreactivity in normal (E), and experimental (F) brain. (G–I) MMP-1-immunoreactivity in experimental brain (details). Scale bars are: 100  $\mu\text{m}$  (C–F), 10  $\mu\text{m}$  (D1, D2, G–I).

was found that the administration of  $\beta$ -amyloid peptide corresponded to the expression of metalloproteinase MMP-1-like immunoreactivity in the same brain areas. In our work, we detected the IR staining in many cases in somata together with primary neurites. The presence of MMP-1-like immunoreactivity in the cells of damaged shape suggests that MMP-1 expression was specifically activated by the A $\beta$  (25–35) injection. Our previous Western blot data (Ierusalimsky and Balaban 2013) – detection of the product corresponding to the pro-form of MMP-1 with this antibody – suggests that increased expression of MMP-1 may be the result of *de-novo* synthesis.

The levels of some A $\beta$  peptidases are decreased in AD brain: neprilysin (Carpentier et al. 2002), plasmin (Ledesma et al. 2000), insulin-degrading enzyme (Bernstein et al. 1999). In contrast, metalloproteinases are activated in AD and after A $\beta$  injection. A $\beta$  (1–40) stimulates MMP-2, MMP-3, and MMP-9 activity in mixed hippocampal and astrocyte cultures (Deb and Gottschall 1996). Levels of MMP-9 were significantly elevated in the plasma of AD patients (Lorenzl et al. 2003). In normal brain, MMP-9 is expressed in pyramidal neurons, and in AD brain MMP-9 was found in hippocampal neurons, neurofibrillary tangles, senile plaques, and vascular walls (Backstrom et al. 1996, Asashina et al. 2001). The astrocytes surrounding amyloid plaques demonstrated an enhanced expression of MMP-2 and MMP-9 in hemizygous double-transgenic mice expressing both human APP and human presenilin 1 (Yin et al. 2006). In AD patients, MMP-3 expression was also detected in hippocampal neurons, around amyloid plaques in the cortex, and in the interstitium of white matter (Yoshiyama et al. 2000).

One of the possible interpretations of these data is the contribution of MMP-2, MMP-3, and MMP-9 to extracellular A $\beta$  clearance by promoting the A $\beta$  catabolism. On the other hand, activation of microglia and astrocytes caused by A $\beta$  deposition in tissues around the plaques is an inflammatory response which might contribute to neuronal death (Selkoe 2001, Rosenberg 2009). The role of MMPs in Alzheimer's disease is complex because of their dual function in the breakdown of amyloid precursor to form the A $\beta$  and in the clearance of A $\beta$  from the brain (Rosenberg 2009). It is known that MMP-9 degrades A $\beta$  *in vitro* (Backstrom et al. 1996). Proteases that degrade the amyloid- $\beta$  peptide are presumed to be important in protecting against Alzheimer's disease (Huang et al. 2008, Hafez et al. 2011).

As MMPs can degrade various proteins of extracellular matrix, the system needs precise control in normal conditions *via* gene transcription, proenzyme activation and by the action of tissue inhibitors of metalloproteinases (Yong et al. 1998). Activation of MMPs with different stimuli is a key feature of many inflammatory and malignant diseases (Yong et al. 1998). It has been shown that MMP-1 levels in cortex were elevated by approximately 50% in AD (Leake et al. 2000). We have not detected MMP-1-like immunoreactivity in hippocampus in control animals, while the A $\beta$  (25–35) injection led to the cell-specific appearance of such immunoreactivity. Our data suggest that MMP-1 in parallel with other MMPs can play a role in the development of Alzheimer's disease.

Thus, administration of  $\beta$ -amyloid peptide induced two parallel processes in the dorsal hippocampus of rats: moderate degeneration of cells, and activation of metalloproteinase of the first type (MMP-1) in these cells. It suggests that several MMPs (including MMP-1) can participate in the development of Alzheimer's disease.

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