Alterations of Emotional and Cognitive Behaviors in Matrix Metalloproteinase-2 and -9-Deficient Mice

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Abstract: Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, and thereby play a crucial role in the remodeling of neural circuits. In the present study, we investigated the role of MMP-2 and MMP-9 in emotional and cognitive function using mice with targeted deletions of the MMP-2 and MMP-9 genes. Emotional behaviors of MMP-9-(-/-) mice but not MMP-2-(-/-) mice were altered, which was manifested in performances in the open-field and elevated plus-arm maze tests. MMP-9-(-/-) mice showed impairments in long-term object recognition memory and conditioned fear memory. MMP-2-(-/-) mice had no deficits in learning and memory. These findings suggest that endogenous MMP-9 play a role in emotional and cognitive behaviors, which may possibly be related to activity-dependent synaptic plasticity and brain development.

Keywords: Matrix metalloproteinase, memory, emotion, behavior.

INTRODUCTION

Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, primarily through the cleavage of extracellular matrix (ECM) proteins and cell-surface components [1]. MMPs constitute a family of enzymes with more than 20 members identified to date, which require Zn^{2+} for their enzymatic activity. Gelatinases (MMP-2 and MMP-9) are capable of cleaving collagen IV and V, laminin, and chondroitin sulfate proteoglycan, which are associated with cell adhesion [1]. MMPs are involved in brain development, because extensive cellular migration and remodeling of the ECM are necessary for neural development [2,3]. Depending on the stage of development, specific and differential expression of MMP and tissue inhibitor of MMP (TIMP) was seen in the cerebellum, which may be related to granular cell migration, arborization of Purkinje cells, and synaptogenesis [4]. Previous studies clearly indicate that a precise knowledge of the relative distribution of the major MMPs is indispensable to delineate their possible role in brain development and plasticity.

The recognition of MMP as a key enzyme in both normal and abnormal nervous system functions represents a rapidly emerging field. Initial studies in this area reported that altered regulation of MMP-2 and MMP-9 was associated with cognitive impairments related to several nervous system disorders. For example, MMP-9 degrades β-amyloid (Aβ) and amyloid plaques [5], and has been implicated specifically in cerebral ischemia [6], kainate-induced neuronal injury [7], and hippocampal long-term potentiation (LTP) and memory [8]. We have also demonstrated that Aβ-induced activation of MMP-9 is related to cognitive impairment induced by Aβ, and that the excessive increase in MMP-9 expression aggravates cognitive impairment [9]. Furthermore, we provided behavioral, neurochemical, and histochemical evidence showing that MMP-2 and MMP-9 are involved in methamphetamine (METH)-induced synaptic plasticity and related behavioral changes by modulating plasmalemmal proteins such as the receptor and transporter [10-12]. Thus, gelatinases are involved in neuronal-activity-dependent synaptic plasticity and cell death in the brain.

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Because of the lack of agents that can selectively inhibit MMP-2 and MMP-9, the physiological roles of endogenous MMP-2 and MMP-9 in brain function such as emotion and memory remain to be determined. To circumvent this problem, we have used mice with targeted deletions of the MMP-2 and MMP-9 genes. We have previously demonstrated that MMP-2 homozygous knock-out [MMP-2-(-/-)] and MMP-9 homozygous knock-out [MMP-9-(-/-)] mice show no difference in locomotor activity compared with their wild-type equivalents [10]. MMP-9 is an inducible protease and expressed in neuronal and grail cells in an activity-dependent manner, while MMP-2 is a constitutive protease. Interestingly, tissue plasminogen activator (tPA) as well as MMP is associated with anxiety-like behavior and memory formation, and some researches demonstrated that tPA in the amygdale promotes stress-induced synaptic plasticity and anxiety-like behavior [13,14]. Thus, it is possible that expression of these proteases can be critical for emotion and memory and that deletion of MMP in mice may exhibit some abnormality in behavioral tests.

In the present study, we evaluated emotional and cognitive behaviors in MMP-2-(-/-) and MMP-9-(-/-) mice. We found that there was some abnormality in performance related to emotionality and long-term memory in MMP-9-(-/-) mice as well as in short-term memory in MMP-2-(-/-) mice. Our findings suggest that the fundamental importance of MMP function in modulating synaptic physiology and plasticity is underscored by behavioral alteration and impairment in emotion and memory displayed in MMP-9-(-/-) mice.

MATERIALS AND METHODOLOGY

Animals

MMP-9-(-/-) mice and equivalent wild-type (FVB/N) mice (10 weeks old) obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at the beginning of the experiments were used. We also used MMP-2-(-/-) mice and equivalent wild-type (C57BL/6J) mice (10–12 weeks old) [15]. These mutant and wild-type mice used in the present study were littermates, and only male mice were used in behavioral test. The animals were housed in plastic cages and kept in a regulated environment (23 ± 1°C, 50 ± 5% humidity) with a 12 h light-dark cycle (lights on at 9:00 am). Food and tap water were available ad libitum.

All experiments were performed in accordance with the Guidelines for Animal Experiments of the Kanazawa University and Nagoya University Graduate School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Open-Field Test

The open field test was carried out as described previously [16], with minor modifications. The open-field used in the study consisted of a circular area with gray walls (60 cm diameter, 60 cm high) and was set in a dark, sound-attenuated room. The floor of the field was divided into one start circle (20 cm diameter) and 18 identical areas so that the animal's ambulation could be measured. The field was divided into inner (40 cm diameter) and outer sectors. A light (100 W) was positioned 100 cm above the center of the floor of the apparatus. Each mouse (n=10 for MMP-9-(-/-) and their wild-type equivalents; n=5 for MMP-2(-/-) and their wild-type equivalents) was placed in the center of the open field. The mice were allowed to freely explore the environment for 10 min. During this time, the ambulation of the mice was measured by counting the number of times that the animals crossed from one area to another. We measured the time spent visiting the inner sector and the number of entries into the inner sector (inner sector visits). The numbers of rearing, climbing, and grooming events were also recorded.

Elevated Plus-Arm Maze Test

The elevated plus-arm maze consisted of two open (30x5 cm) and two closed arms (30x5x25 cm) emanating from a common central platform (5x5 cm) to form a plus shape. The entire apparatus was elevated to a height of 40 cm above floor level. Testing commenced by placing a mouse (n=10 for MMP-9-(-/-) and their wild-type equivalents; n=5 for MMP-2(-/-) and their wild-type equivalents) on the central platform of the maze facing an open arm, and a standard 5-min test duration was employed. Conventional parameters consisted of the numbers of open and closed arm entries and the time spent in the open arms.

Novel-Object Recognition Test (NORT)

The NORT was carried out as described previously [18,19]. The experimental apparatus consisted of a Plexiglas open-field box (30x30x35 cm), with a sawdust-covered floor. The apparatus was located in a sound-attenuated room and was illuminated with a 20 W bulb.

In a standard procedure, the NORT consisted of three sessions: habituation, training, and retention. Each mouse (n=10 for MMP-9-(-/-) and their wild-type equivalents; n=8-10 for MMP-2(-/-) and their wild-type equivalents) was individually habituated to the box, with 10 min of exploration in the absence of objects for 3 consecutive days (habituation session, days 1–3). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore the box for 10 min (day 4). The objects were constructed from a golf ball, a wooden column, and a wall socket, which were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was facing the object and/or it was touching or sniffing the object. The time spent exploring each object was recorded, and the mice were immediately returned to their home cages after training. The animals were placed back into the same box once, either 1 or 24 hr after the training session, to assess short-term and long-term memory, respectively (retention session). During the retention sessions, one of the familiar objects used during training had been replaced with a novel object. The animals were then allowed to explore freely for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index in the retention session, a ratio of the amount

of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as the ratio of the time spent exploring the object that was replaced by the novel object in the retention session over the total exploring time.

Cued and Contextual Fear Conditioning Tests

Cued and contextual fear conditioning tests were performed in accordance with previous reports [17], with minor modifications. Freezing behavior, which shows mice's head, arms and legs not moving, was measured by stop-watch. For measuring basal levels of freezing response (preconditioning phase), mice (n=8-11) were individually placed in a neutral cage (a block Plexiglas box with abundant wood tips, 30x30x35 cm) for 1 min, then in a conditioning cage (a transparent Plexiglas box, 30x30x35 high cm) for 2 min. For training (conditioning phase), mice were placed in the conditioning cage, then a 15 s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea Co., Ltd.). This procedure was repeated 4 [MMP-2-(-/-)] or 8 [MMP-9-(-/-)] times with 15 s intervals. Cued and contextual tests were carried out 1 day after fear conditioning. For the contextual test, mice were placed in the conditioning cage and the freezing response was measured for 2 min in the absence of the conditioned stimulus. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus.

Statistical Analysis

All data were expressed as the mean ± SE. Statistical significance was determined using Student's t-test for two-group comparisons, or a one-way analysis of variance (ANOVA), followed by the Fisher' LSD test for multigroup comparisons. P values less than 0.05 were taken to indicate statistically significant differences.

RESULTS

Performance in Open-Field and Elevated Plus-Arm Maze Tests by MMP-9-(-/-) and MMP-2-(-/-) Mice

There was a clear strain difference of performance in open-filed and elevated plus-arm maze test between FVB/N mice and C57BL/6J mice (Fig. **1** and Fig. **2**). It was mani-

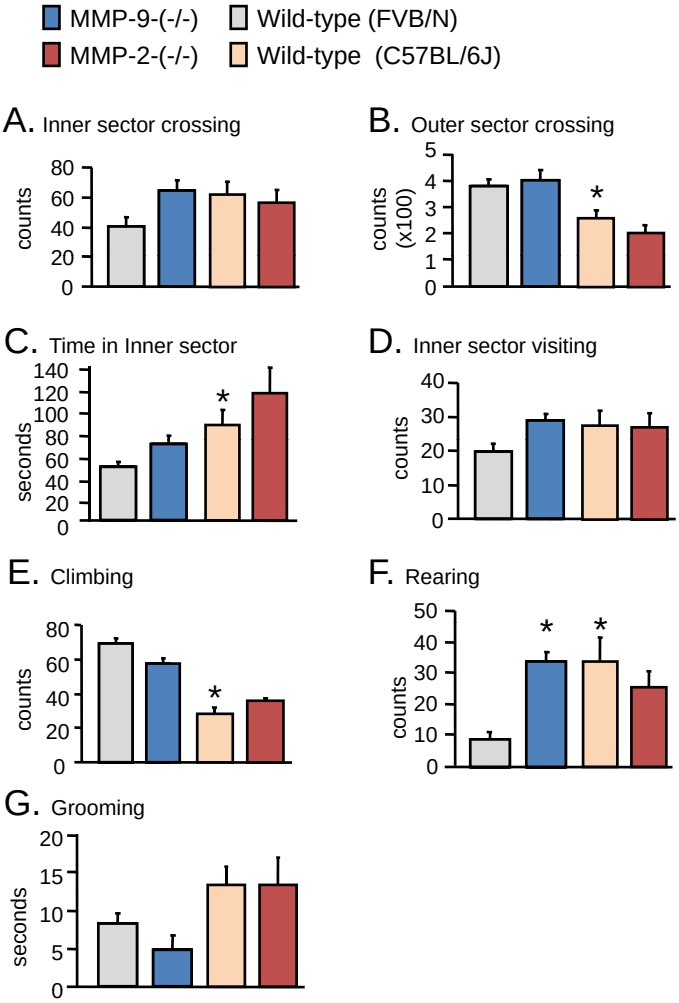

Fig. (1). Performance in the open-field test by MMP-2-(-/-) mice and MMP-9-(-/-) mice. In the open-field test, each mouse was allowed to freely explore the environment for 10 min. Values are the mean±S.E. (n=10 for MMP-9-(-/-) and their wild-type equivalents, n=5 for MMP-2(-/-) and their wild-type equivalents). *p<0.05 *vs*. wild-type (FVB/N) mice.

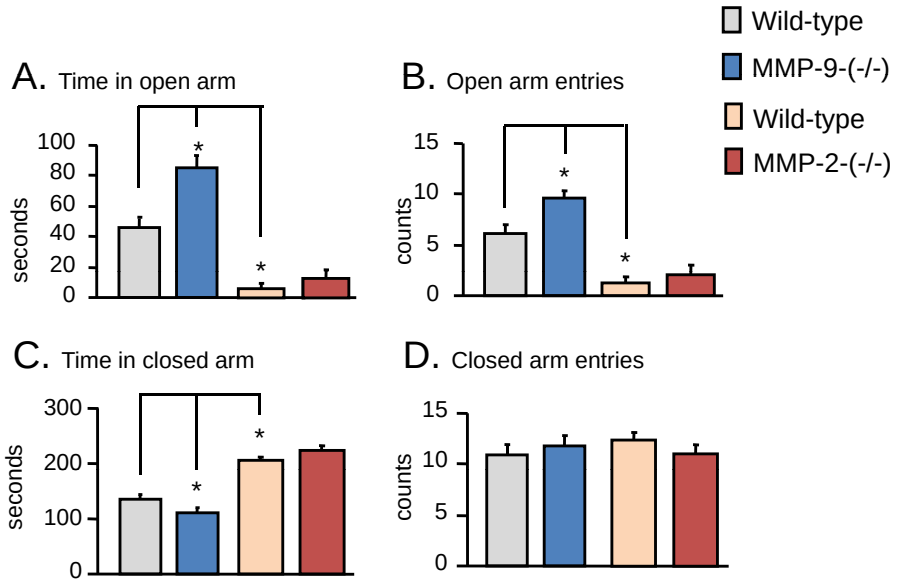

Fig. (2). Performance in the elevated plus-arm maze test by MMP-2-(-/-) mice and MMP-9-(-/-) mice. In the elevated plus-arm maze test, each mouse was allowed to freely explore the maze for 5 min. Values are the mean±S.E. (n=10 for MMP-9-(-/-) and their wild-type equivalents, n=5 for MMP-2(-/-) and their wild-type equivalents). *p<0.05 *vs*. wild-type (FVB/N) mice.

fested by the changes in outer sector crossing (Fig. **1B**, $F(3,26)=7.67$, $p<0.05$), time in inner sector (Fig.**1C**, $F(3,26)=6.34$, $p<0.05$), climbing (Fig.**1E**, $F(3,26)=9.57$, $p<0.05$), rearing (Fig.**1F**, $F(3,26)=6.82$, $p<0.05$) in the open-field test as well as the changes in time in open arm (Fig. **2A**, $F(3,26)=27.7$, $p<0.05$), open arm entries (Fig. **2B**, $F(3,26)=19.7$, $p<0.05$), and time in closed arm (Fig. **2C**, $F(3,26)=33.2$, $p<0.05$) in the elevated plus-arm maze test. Because it is obvious that we cannot compare the effects of targeted deletions of MMP-2 and MMP-9 genes on emotional behaviors, the comparison was made with the same strain.

When MMP-9-(-/-) mice were exposed to a novel environment under mild stressful conditions in the open-field test, they showed a significantly increased number of rearing events compared with wild-type mice (Fig.**1F**, $p<0.05$). The mutant mice showed a tendency to explore the inner sector of open-filed more than did wild-type mice, as indicated by an increased inner sector crossing (Fig. **1A**), time in the inner sector (Fig. **1C**) and inner sector visiting (Fig. **1D**). But these alterations were not statistically significant. There was no difference in any behavioral events between wild-type and MMP-2-(-/-) mice in the open-field test (Fig. **1**).

To further evaluate emotional change, MMP-9-(-/-) and MMP-2-(-/-) mice were subjected to the elevated plus-arm maze test. The time spent in open arms and open arm entries by MMP-9-(-/-) mice was significantly longer than those by wild-type mice while that in closed arms was shorter than that by wild-type mice (Fig.**2A**, $F(3,26)=27.7$, $p<0.05$; **2B**, $F(3,26)=19.7$, $p<0.05$; **2C**, $F(3,26)=33.2$, $p<0.05$). In contrast, there was no difference in performance in the elevated plus-maze test between wild-type and MMP-2-(-/-) mice (Fig. **2**).

Alternation Behavior in Y-Maze task in MMP-9-(-/-) and MMP-2-(-/-) Mice

We evaluated short-term working memory in MMP-9-(-/-) and MMP-2-(-/-) mice in a Y-maze test. One-way ANOVA revealed that there was a significant difference in the numbers of arm entries ($F(3,28)=5.78$, $p<0.05$), but not in spontaneous alternation behavior (%) ($F(3,28)=2.51$, $p>0.05$) among 4 groups of animals. The post-hoc analysis of arm entries indicated that there were no differences between FVB/N mice and C57BL/6J mice or between mutant mice and the respective wild-type mice (data not shown).

Recognition Memory in MMP-9-(-/-) and MMP-2-(-/-) Mice

We used a novel-object recognition test to assess short-term and long-term recognition memory [18,19]. In the 1-hr retention session, MMP-9-(-/-) and MMP-2-(-/-) mice exhibited levels of exploratory preference and exploration time for the novel objects similar to those of their respective wild-type mice, suggesting no impairment of short-term memory (data not shown).

When retention performance was tested 24 hr after the training session, one-way ANOVA indicated a significant difference in the level of exploratory preference for the novel objects among 4 groups of animals (Fig. **3**, $F(3,34)=7.19$, $p<0.05$). Post-hoc analysis indicated that the exploratory preference for the novel objects in MMP-9-(-/-) mice was significantly decreased compared with that in the wild-type mice ($p<0.05$). There were apparent differences in total exploration time during the training (Fig. **3A**, $F(3,34)=18.1$, $p<0.05$) and retention sessions (Fig. **3A**, $F(3,34)=35.9$, $p<0.05$) between FVB/N mice and C57BL/6J mice, but no difference was observed between mutant mice and the respective wild-type mice. There was no difference in exploratory preference and exploration time in training and retention session between MMP-2-(-/-) and wild-type mice (Fig. **3B**). These results suggest that MMP-9-(-/-) mice but not MMP-2-(-/-) mice have the deficit in long-term memory retention but that the memory acquisition (learning) and short-term memory of MMP-9-(-/-) mice are not impaired.

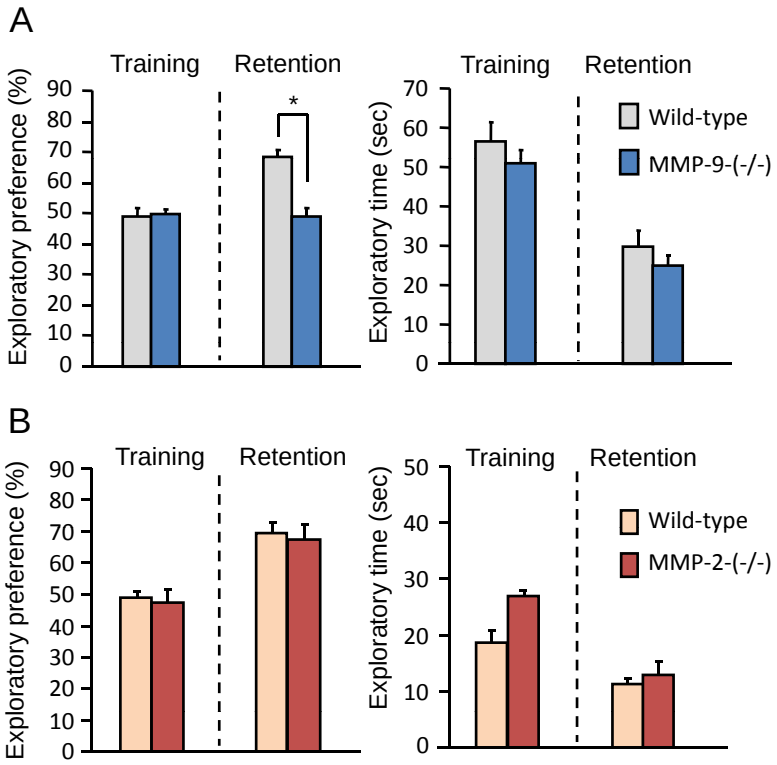

Fig. (3). Performance in the long-term NORT by MMP-9-(-/-) (**A**) and MMP-2-(-/-) (**B**) mice. The retention session was carried out 24 hr after the training. Values are the mean±S.E. (n=10 for A, n=8-10 for B). *p<0.05 *vs.* wild-type mice.

Conditioned Fear Memory in MMP-9-(-/-) and MMP-2-(-/-) Mice

We evaluated associative learning in a conditioned fear learning test. In the preconditioning phase, all groups of mice hardly showed any freezing response. Because different protocols for the training were used in FVB/N mice and C57BL/6J mice, statistical comparison was made between the mutant mice and their respective wild-type mice.

MMP-9-(-/-) mice exhibited less freezing response than their wild-type equivalents in the cued (Fig. **4A**, p<0.05) and contextual test (Fig. **4B**, p<0.05), indicating an impairment of associative learning. There was no difference in the cued and contextual freezing responses between MMP-2-(-/-) and wild-type mice (Fig. **4**). There was an obvious difference in the minimal current required to elicit flinching/running, jumping, or vocalization between FVB/N mice and C57BL/6J mice, but no alterations were found between mutants and their respective wild-type equivalents (MMP-9-(-/-): 0.31±0.02 mA, wild-type: 0.33±0.01 mA; MMP-2-(-/-): 0.16±0.03 mA, wild-type: 0.19±0.02 mA).

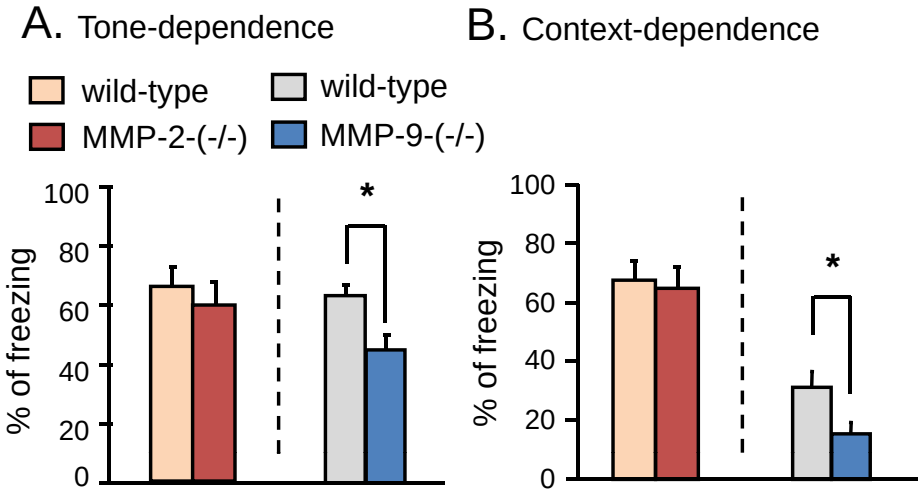

Fig. (4). Performance in the conditioned fear learning test by MMP-9-(-/-) and MMP-2-(-/-) mice. The retention session was carried out 24 hr after the training. Cue-dependent (**A**) and context-dependent (**B**) freezing times were measured. Values are the mean±S.E. (n=8-11). *p<0.05 *vs.* wild-type mice.

DISCUSSION

The present study investigated the role of MMP-2 and MMP-9 in emotional and cognitive function by assessing the performance of mice with targeted deletions of the MMP-2 and MMP-9 genes in various behavioral tasks. MMP-9-(-/-) mice but not MMP-2(-/-) mice showed a behavioral response indicating reduced anxiety upon exposure to mild stressful situations. The emotional changes were manifested by increased numbers of rearing in the open-field test, and increased time spent in the open arms and more open arms entries in the elevated plus-arm maze test. Accordingly, it is plausible that endogenous MMP-9 may be involved in the modulation of emotional behavior. However, a previous report showed that MMP inhibition disrupted reconsolidation of the fear memory, which is related to emotionality in a reactivation-dependent manner, and that the reduced freezing behavior was not due to a decrease in general anxiety levels, since FN-439, a broad-spectrum metalloproteinase inhibitor, had no effect on the time spent in open arms or on the numbers of open arm entries in an elevated plus-arm maze task, suggesting that MMP may be involved in fear memory, but not anxiety-like fear [20]. Therefore, further experiments are necessary with various anxiety tests, since an anxiogenic behavioral response could be tied to specific stimuli, influenced by motor factors, and situation-specific. Additionally, it is well known that phenotypic changes in behavior of mutant mice are dependent, at least in part, on the genetic background, C57BL/6J and FVB/N. Thus, we have to interpret the results in the present study with caution.

MMP-9-(-/-) mice showed an impairment of long-term object recognition and fear memory, while spontaneous alternation behavior in the Y-maze test and short-term memory were intact. These results imply that MMP-9 plays a role in the formation of long-term but not short-term memory. In agreement with our results, the infusion of an MMP inhibitor into the dorsal hippocampus was found to disrupt acquisition of spatial memory in Morris's water maze [21]. Hippocampal MMP-9 expression is increased transiently during water maze acquisition, and inhibition of MMP activity with MMP-9 antisense oligonucleotides and MMP inhibitor altered LTP and prevented acquisition of spatial memory in Morris's water maze [22]. Furthermore, Nagy *et al*. [8] showed that MMP-9 null-mutant mice display a significant deficit in long-term hippocampus-dependent memory for context but not cued conditioning compared with their wild-type equivalents. These results indicate that changes in MMP function are critical in synaptic plasticity and hippocampal-dependent memory and that compromising the ability of the dorsal hippocampus to reconfigure ECM molecules by inhibiting MMP activity interferes with appropriate spatial memory acquisition. In contrast to the report by Nagy *et al.*, our findings showed that hippocampus-independent cued fear memory was also disrupted in MMP-9-(-/-) mice compared with that in wild-type mice, indicating that the functioning of the corticolimbic system including the amygdala is disrupted in MMP-9-(-/-) mice. The discrepancy between our findings and those of previous studies may reflect differences in the methodology of the fear memory test and the types of mutant mice used.

Activity-dependent changes in the organization of ECM are considered to alter synaptic architecture and physiology in a way that changes the efficiency of synaptic transmission. Recent study has identified that MMP-9 as a physiological regulator of N-methyl-$_D$-aspartate (NMDA) receptor-dependent synaptic plasticity and memory. This mechanism of MMP-9 action on NMDA receptors is not mediated by change in overall ECM structure nor by direct cleavage of NMDA receptor subunits, but rather through an integrin beta1-dependent pathway [23]. The NMDA receptor is an important mediator of synaptic plasticity and plays a central role in the neurobiological mechanisms of emotionality, as well as learning and memory [24]. In fact, we have demonstrated that MMP-9 plays a role in METH-induced behavioral sensitization and reward as well as dopamine release through modulation of the function of dopamine receptors and transporters [10,11].

MMP, by focalized and controlled proteolysis, may be crucial in determining the hierarchy of processes involved in brain development and plasticity. In the cerebellum, there is differential and spatiotemporal expression of MMP-2 and MMP-9 during its development [4]. These regional and cellular expression patterns are evidence for tightly regulated proteolysis-mediated alteration of ECM components, which might be related to the migration of granular precursors and Purkinje cell arborization. The spatiotemporal regulation of MMPs may be crucial in brain maturation and plasticity. Accordingly, we cannot exclude the possibility that neuroadaptation and compensatory mechanisms for the targeted deletions of the MMP-2 and MMP-9 genes may contribute to the altered emotional and cognitive behaviors in the mutant mice, although histochemical examinations revealed no major defects in brain structure.

In conclusion, the present study demonstrates that mice with targeted deletions of the MMP-9 gene show deficits in emotional and cognitive behaviors while the deletion of the MMP-2 gene has no effect on these behaviors. These results suggest that endogenous MMPs, especially MMP-9, have a role in the regulation of emotion and memory.

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ABBREVIATIONS

Aβ = β-amyloid

ECM = Extracellular matrix

LTP = Long-term potentiation

METH = Methamphetamine

MMPs = Matrix metalloproteinases

NORT = Novel-object recognition test

NMDA = *N*-methyl-$_D$-aspartate

TIMP = Tissue inhibitor of MMP

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