MMP-7 cleaves the NR1 NMDA receptor subunit and modifies NMDA receptor function

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ABSTRACT Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that play a role in the inflammatory response. These enzymes have been well studied in the context of cancer biology and inflammation. Recent studies, however, suggest that these enzymes also play roles in brain development and neurodegenerative disease. Select MMPs can target proteins critical to synaptic structure and neuronal survival, including integrins and cadherins. Here, we show that one member of the MMP family, MMP-7, which may be released from cells, including microglia, can target a protein critical to synaptic function. Through analysis of extracts from murine cortical slice preparations, we show that MMP-7 cleaves the NR1 subunit of the N-methyl-D-aspartate (NMDA) receptor to generate an N-terminal fragment of ~65 kDa. Moreover, studies with recombinant protein show that MMP-7-mediated cleavage of NR1 occurs at amino acid 517, which is extracellular and just distal to the first transmembrane domain. Data suggest that NR2A, which shares sequence homology with NR1, is also cleaved following treatment of slices with MMP-7, while select AMPA receptor subunits are not. Consistent with a potential effect of MMP-7 on ligand binding, additional experiments demonstrate that NMDA-mediated calcium flux is significantly diminished by MMP-7 pretreatment of cultures. In addition, the AMPA/NMDA ratio is increased by MMP-7 pretreatment. These data suggest that synaptic function may be altered in neurological conditions associated with increased levels of MMP-7.—Szklarczyk, A., Ewaleifoh, O., Beique, J.-C., Wang, Y., Knorr, D., Haughey, N., Malpica, T., Mattson, M. P., Huganir, R., Conant, K.

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MMPs are zinc-dependent endopeptidases that were named for their ability to act on proteins of the extracellular matrix but are now known to process nonmatrix proteins, including soluble molecules and cell surface receptors (1). These enzymes are expressed in the central nervous system and are known to play a role in normal brain development and physiology (2). For example, MMP-9 plays a role in cerebellar development (3), while MMP-9 and MMP-12 contribute to developmental myelination through their ability to process IGF binding proteins (4). Though physiological expression and release of MMPs are likely important to normal central nervous system (CNS) function, pathological expression with excess proteolysis may be detrimental. MMP levels are elevated in association with a variety of inflammatory and degenerative diseases of the central nervous system, including Alzheimer’s disease, HIV dementia, stroke, amyotrophic lateral sclerosis, and multiple sclerosis (5–9), and evidence from animal studies suggests that they play a role in the pathology of such conditions. For example, reduced MMP-9 activity is protective with respect to ischemia or spinal cord trauma (10–12).

In vitro studies also support the possibility that MMPs contribute to neuronal dysfunction in the setting of CNS inflammation. Several studies have shown that MMPs may adversely influence neuronal survival, and a variety of mechanisms have been implicated (13–15). MMPs cleave integrin binding matrix proteins and may thus stimulate cell death through detachment. MMPs can also interact with cell surface receptors, including integrins (16), and at least one MMP can activate a G protein-coupled thrombin receptor, proteinase-activated receptor-1 (17). Activation of PAR-1 has been linked to the death of motor neurons (18).

Effects of MMPs on the neuronal synapse have been less well studied. Nonetheless, emerging evidence suggests that these enzymes might also influence synaptic function, and that such effects may be beneficial and/or harmful. Among the targets of MMPs are beta dystroglycan (19) and synaptic adhesion molecules, 1 Correspondence: Johns Hopkins University, Department of Neurology, Pathology Bldg. Rm. 625, 600 N. Wolfe St., Baltimore, MD 21287, USA. E-mail: kconant@mail.jhmi.edu doi: 10.1096/fj.07-101402
including syndecans and cadherins (20–22). In addition, MMP-7 has been shown to influence the morphology of dendritic spines (23), and MMP-9 has been linked to altered long-term potentiation (24, 25). In one study, this effect was integrin dependent and may have involved MMP-9-mediated generation of a matrix fragment that could influence the phosphorylation of select NMDA receptor (NMDAR) subunits (24).

In the present study, which followed an initial interest in whether select MMPs might act on PAR-1 and/or select integrins to alter NMDAR subunit phosphorylation (26), we have investigated the possibility that an MMP might cleave select NMDAR subunits. We have focused on MMP-7 because this MMP lacks a C-terminal hemopexin domain, making it less susceptible to binding and inhibition by endogenous inhibitors of MMPs [tissue inhibitors of MMPs (TIMPs)]. Moreover, MMP-7 is known to have a relatively broad substrate range and to be elevated in several inflammatory diseases of the CNS (6, 7).

Here, we show that MMP-7 can cleave NR1, an obligate subunit of the NMDAR. Cleavage of NR1 occurs at a site within the ligand binding domain. We also show that pretreatment of cortical neurons with MMP-7 impairs NMDA-stimulated calcium flux. Together, these studies lend support to the possibility that neurotransmission may be altered in conditions characterized by elevated CNS levels of MMP-7.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant MMP-7 (human, active, cat. no. 444270) was from Calbiochem/EMD Biosciences (San Diego, CA, USA) unless otherwise indicated. MMP-1 was from RKD Systems (Minneapolis, MN, USA; 901MP), while MMP-2 and MMP-9 were from Calbiochem (PF023 and PF024). Of note, although the sequence of a given MMP may vary depending on the species of origin, substrate specificity is usually conserved, so that an MMP from one species will typically cleave a substrate from its own species and from a variety of others. The MMP inhibitor (MMPI), GM6001, was also from Calbiochem (364205) and used at a concentration of 25 μM, unless otherwise indicated. Chlormazine was from Sigma-Aldrich (St. Louis, MO, USA). Cyclosporine A and calpain inhibitor V were from Calbiochem. Anti-NR1 N-terminal antibodies were supplied by R.H., and had been prepared against amino acids 229–247 (Ab1) or 436–450 (Ab2) as indicated. The anti-NR1 C terminal antibody was from Millipore (06–314). Anti-gluR antibodies were also from the laboratory of R.H., and anti-NR2A was from Chemicon (Temecula, CA, USA; MAB5216). Recombinant NR1 ligand binding domain was kindly provided to us by Drs. Y. Yao and M. Mayer at the National Institutes of Health (Bethesda MD, USA). This recombinant rat construct has been previously described (27) and includes two extracellular regions from Met394 to Lys544 (S1) and from Arg663 to Ser-800 (S2); the S1 and S2 segments are connected by a dipeptide Gly-Thr linker, and there is a polyhistidine tag followed by a thrombin proteolysis site on the amino terminus of S1 (28).

Acute cortical slices

Acute slice cultures (200 μm) were prepared from 8- to 10-wk-old C57/B6 mice or Sprague-Dawley rats using a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). The protocol was based on Grosshans et al. (29). Dissection of hippocampi was performed in D1 medium (10 mM HEPES, 0.3% glucose, 0.75% sucrose, pH=7.4). After separation, slices were placed in 24-well plates, 5 slices per single well, in 0.5 ml of serum-free MEM, and transferred to a cell incubator (37°C) for 30 min to 6 h.

Dissociated neuronal cultures

Primary cortical neurons were derived from E18 rats (Sprague-Dawley; Taconic, Germantown, NY, USA) and prepared as described (30). Briefly, tissue was dissociated by gentle trituration in a calcium-free Hank’s balanced salt solution and was centrifuged at 1000 g. Cells were resuspended in minimal essential medium containing 10% fetal bovine serum (FBS) and 1% antibiotic solution (10 U of penicillin/ml, 10 mg streptomycin/ml, and 25 μg amphotericin B/ml; Sigma). Neurons were then plated at a density of 10^6 cells/ml on poly-D-lysine-coated tissue culture ware. Three hours after plating, the medium was replaced with serum-free Neurobasal medium containing 1X B-27 supplement (Life Technologies, Inc., Rockville, MD, USA). Cultures were used between 10 and 14 days in vitro. Immunofluorescent staining of similarly prepared cultures for MAP-2 (neurons) and glial fibrillary acidic protein (GFAP) (astrocytes) showed that cultures were >90% neurons, with the remainder predominantly astrocytes.

Preparation of lysates

Following a wash and aspiration of serum-free medium, lysates were prepared via the addition of lysate buffer to cultured cells or slices [50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% sodium dodecyl sulfate (SDS); 1% Nonidet P-40, 0.5% sodium deoxycholate; 0.2 mM phenylmethylsulfonyl fluoride (PMSF); 0.5 mM dithiothreitol (DTT); and 1X protease inhibitor cocktail (Sigma P8340)]. The MMP activity inhibitor GM6001 was also added to the lysis buffer (10 μM). The mixture was placed into an Eppendorf tube, sonicated for 10 s, kept on ice for 20 min, and then spun at 14,000 rpm for 15 min at 4°C in an Eppendorf centrifuge (Eppendorf, Westbury, NY, USA). Supernatants were then saved and used in Western blot experiments.

Western blot analysis

Western blot of cell lysates was performed using 20 μg of protein per lane, as determined by the Pierce BCA assay (Pierce, Rockford, IL, USA). Before analysis, samples were mixed with sample buffer containing 5% β-mercaptoethanol and boiled for 5 min at 95°C. Electrophoresis was performed on a 4–15% Tris-glycine polyacrylamide gradient gel (Bio-Rad, Hercules, CA, USA). Following electrophoretic transfer of the protein to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), the membrane was blocked in 5% nonfat dry milk in TBST (150 mM NaCl, 100 mM Tris base, 0.1% Tween 20, pH 7.6) buffer for 1 h. The blot was then probed with the indicated primary antibody, at a dilution recommended by the manufacturer, for 1.5 h at room temperature. After washing the membrane 3X (15 min each) in TBST buffer, it was incubated with an appropriate secondary antibody for 0.5 h at room temperature. The membrane was then washed again in TBST buffer, and immunoreactive bands...
were visualized using electrochemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Western blot of recombinant protein digests was performed similarly, except that the entire reaction, rather than 20 μg of total protein, was analyzed. In these experiments, 500 ng of the recombinant protein had been incubated with enzyme, and the digestion had been carried out for 30 min in 50 μl of buffer (50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 1 mM ZnCl₂, pH 7.5) at 37°C.

**Immunocytochemistry**

Neuronal cultures and slices were treated with either vehicle or MMP-7 and/or GM6001, as indicated, in serum-free medium. Cells were washed 3× with fresh changes of medium, and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) for 20 min at 4°C. Primary antibody (anti-NR2A; Upstate, Lake Placid, NY; MAB5216) was applied in phosphate-buffered saline (PBS) with 5% normal goat serum (Vector, Burlingame, CA, USA) and 0.1% Triton X-100 (Sigma). The staining was carried out for 24 h at 4°C. After washing in phosphate-buffered saline with 0.1% Tween (PBST), immunolabeling was continued with an anti-mouse secondary antibody coupled to red Alexa 568 (Molecular Probes, Eugene, OR, USA; 1:500). After incubation for 2 h at room temperature with the secondary antibody, cultures were washed in PBS and mounted in ProLong (Molecular Probes). Images were taken with a Zeiss 510 confocal microscope (Carl Zeiss, Jena, Germany).

For analysis, mean gray values for randomly selected fluorescent puncta (which represent clusters of NMDAR2A) were subsequently obtained and evaluated using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA). A minimum of 30 puncta from 5 primary dendrites was analyzed. Values were then expressed as means ± sd (arbitrary units). For analysis of immunocytochemistry performed with slices, fluorescent gray value units were derived from fields within a slice. Five random fields within the molecular layer of the dentate gyrus were evaluated.

**Peptide sequencing**

The products of recombinant NR1/MMP-7 reactions were resolved on a 4–15% Tris-glycine polyacrylamide gradient gel (Bio-Rad, Hercules, CA), and then transferred to a PVDF membrane (Bio-Rad). The conjugate and MMP-7 were run on the same gel and similarly transferred so that bands unique to the digests could be identified. Following protein transfer, the PVDF membrane was stained with sequencing grade Coomassie blue (Bio-Rad; 1610436). Appropriate bands were identified and cut out for N-terminal sequencing.

N-terminal sequencing was performed with the Perkin-Elmer/Applied Biosystems Procise Protein Sequencing System (Applied Biosystems, Foster City, CA, USA). This method is compatible with samples that have been electroblotted onto PVDF.

**Calcium imaging**

Cytosolic calcium ([Ca²⁺]i) was determined using the ratiometric Ca²⁺-specific fluorescent probe Fura-2AM, as described previously (31). Neurons were incubated with 1 μM Fura-2AM for 15 min at 37°C in neurobasal medium, then washed with Locke’s buffer (154 mM NaCl, 3.6 mM NaHCO₃, 5.6 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 2.3 mM CaCl₂, 10 mM glucose) to remove extracellular Fura-2 and were incubated at 37°C for 10 min to allow complete de-esterification of the probe. Coverslips containing Fura-2-loaded cells were maintained at 37°C in a recording chamber and perfused with Locke’s buffer at the rate of 2 ml/min (Warner Instruments Inc., Hamden, CT, USA). Image pairs were acquired every 1.0–5.0 s.

**Preparation of slices for electrophysiology**

Rats aged 17–25 days were anesthetized with halothane (by inhalation) and euthanized by decapitation. The brain was quickly removed and cooled in ice-cold Ringer solution of the following composition (mM): 119 cholineCl, 2.5 KCl, 4.3 MgSO₄, 1 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃ and 22 glucose, bubbled to saturation with 95% O₂/5% CO₂. Coronal slices (300 μm thick) were cut using a vibratome (VT100S; Leica, Wetzlar, Germany), and transferred to a holding chamber where they were allowed to recover for 1 h at 35–35°C in normal Ringer solution containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃ and 11 glucose. After 1 h, the holding chamber was placed at room temperature until recordings.

**Whole-cell recordings**

Hippocampal CA1 pyramidal neurons were targeted for whole-cell patch-clamp recording using differential interference contrast imaging on a fixed-stage upright microscope (Axioskope; Carl Zeiss). Electrical signals were recorded using an Axopatch 200, an Axopatch 200B or a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, USA). All recordings were carried out at room temperature. Recordings were filtered at 2 kHz, digitized at 10 kHz, and acquired with Clampex (Axon Instruments). Liquid junction potentials and voltages were left uncompensated. The intracellular solution used was composed of (mM) 77 cesium gluconate, 10 tetrasodium BAPTA, 5 TEA-Cl, 3 CaCl₂, 20 HEPES, 4 MgATP, 0.5 GTP, 5 QX-314 and 10 Na₂ phosphocreatine. The pH was adjusted to 7.3 and osmolality to 280–290 mosmol. In all experiments, Ringer was supplemented with 30 mM (−)bicineulline. Access resistance was continuously monitored during the experiments by delivering a 3–5 mV hyperpolarizing voltage step 180 ms before the delivery of the stimulus pulse. Experiments were discarded if the access resistance changed by more than ~25%.

Evoked excitatory postsynaptic currents (eEPSCs) were obtained by placing a bipolar stainless steel stimulating electrode in the stratum radiatum. To estimate the AMPA/NMDA ratio, we directly estimated the amplitude of AMPAR and NMDAR current at 40 mV by virtue of their different kinetics, as described previously (32). The time window to measure the AMPAR current at +40 mV was determined from the eESPC trace obtained at −70 mV. The time window to measure the NMDAR current at +40 mV was set at 3× decay time constant of AMPAR current at −70 mV (τ_{AMPAR}, usually around 10–12 ms) following the peak of the AMPAR response at −70 mV.

**Statistics**

Statistical calculations for immunocytochemistry and calcium imaging values were performed through GraphPad Prism (GraphPad, San Diego, CA, USA). ANOVA with Tukey’s post hoc testing was utilized. Statistical comparison of the AMPA/NMDA ratio data involved only two conditions, control and MMP-7, and therefore Student’s t test was used to assess statistical significance.
RESULTS

Treatment of acute murine cortical slice cultures with MMP-7 generates a potential NR1 cleavage fragment

Previous work supports the possibility that certain proteinases may influence NMDA receptor structure and/or function (33). For example, thrombin may act on proteinase-activated receptors to influence NMDAR subunit phosphorylation (34). In addition, tissue plasminogen activator binds to and cleaves NR1 subunits so as to influence NMDA stimulated calcium flux and cell death (35). To determine whether MMP-7, a proteinase known to be elevated in select inflammatory diseases of the CNS (6, 7), might influence NMDAR integrity, we used Western blot analysis to examine the NR1 NMDAR subunit in MMP-7 treated acute murine slice cultures.

Cultures were incubated for 6 h (Fig. 1A) or for 0.5, 2, or 5 h (as indicated, Fig. 1B) in the presence or absence of 50 nM of the indicated MMP. Extracts were subsequently prepared, and Western blot analysis was performed using an antibody to NR1 amino acids 229–247. A potential N-terminal cleavage product (lowest molecular mass band) was present (Fig. 1A, B). Overall, a potential cleavage product was observed in 5/5 slice preparations treated with MMP-7 and 0/7 that were untreated or treated with other MMPs. Although we show results from 2 experiments, we saw an N-terminal NR1 fragment of ~65 kDa in a total of 5 independent experiments, including a control experiment shown to follow (Fig. 1C). In these experiments, the fragment showed varied intensity but was present only in lysates from MMP-7-treated slices. The varied intensity of immunoreactive bands likely reflects differential abundance and accessibility of NR1 in the different slices, and retention of the potential cleavage fragment likely represents association with cell matrix or other proteins integral to the slice.

To further assess potential NR1 cleavage by MMP-7, we also examined NR1 integrity using an antibody to the C-terminal domain (Fig. 1C). In this experiment, pretreatment of slices with 50 nM MMP-7 for 6 h was associated with the generation of potential C-terminal degradation products, as indicated by an increase in bands and band intensity in the lower molecular mass range (25–75 kDa). Again, the arrow indicates a band having apparent molecular mass of full-length NR1.

MMP-7 cleaves recombinant NR1 ligand binding domain

The region of NR1 that is N-terminal to the first transmembrane domain is 561 amino acids in length. On the basis of the molecular mass of these 561 residues, and thus not accounting for potential glycosylation, the total molecular mass would be ~73 kDa (72.8). Because data shown in Fig. 1 suggest that MMP-7 may generate an N-terminal cleavage fragment of ~65 kDa, cleavage likely occurs distal to the first transmembrane domain and might involve the S1 portion of the ligand binding domain. We thus investigated the possibility that MMP-7 might cleave a recombinant NR1 ligand binding domain construct that includes amino acids Met394 to Lys544 (S1) and Arg663 to Ser-800 (S2).

As shown in Fig. 2, MMP-7 was associated with cleavage of this recombinant construct. Digestion of rLBD by MMP-7 gave rise to fragments with an apparent molecular mass between 10 and 15 kDa. N-terminal sequencing showed that one of the newly generated fragments (arrowhead, Fig. 2B) had an N terminus beginning with LTINNE, suggesting that cleavage occurs between Pro 516 and Lys 517. Cleavage at this site is consistent with the results shown in Fig. 1, in that a product of 516 amino acids would be expected to run at 69.7 kDa, a molecular mass fairly close to what we observed. In addition, cleavage at Lys 517 is of interest in that Pro 516 may contribute to ligand binding by forming a hydrogen bond to bound agonists (36).
MMP-7 affects a loss of full-length NR2A

NMDARs are heterotetramers containing two NR1 subunits in combination with two regulatory subunits (NR2A-D, NR3A-C). Most native NMDARs function as heterotetrameric associations of 2 NR1 and 2 NR2 subunits. NR2A containing NMDARs are of particular interest in that they associate with PSD95 and show relatively high expression at the mature synapse.

Beginning with amino acid 517, the NR1 sequence starts with LTIN. Beginning with amino acid 512, NR2A shares this sequence. We thus investigated the possibility that MMP-7 might cleave NR2A. As shown in Fig. 3A, we show that MMP-7 treatment of murine cortical slice preparations is associated with a loss of full-length NR2A immunoreactivity. The loss is relatively profound and may reflect the higher surface to cytoplasmic ratio of NR2A, as compared to NR1.

In Fig. 3B, we also show that full-length NR2A is diminished in rat cortical slice preparations following their exposure to 50 nM MMP-7. Moreover, because the loss of full-length NR2A could reflect cleavage in the extracellular domain, or endocytosis and internal processing of the full-length receptor subunit, we also tested various inhibitors for their potential to influence the MMP-7-associated loss of NR2A immunoreactivity. As shown in Fig. 3B, however, this loss was not diminished by pretreatment of cultures with chlorpromazine, which is thought to inhibit clathrin-mediated endocytosis of NMDA receptor subunits through effects on the adaptor protein AP2 (37). Nor was this loss diminished by pretreatment of cultures with cyclosporine A (CsA), an inhibitor of the calcium-activated phosphatase calcineurin, which may play a role in loss of surface subunit expression (38). And finally, inhibition of calpain, which may play a role in NMDAR subunit proteolysis following its endocytosis, was without effect (39). In a separate experiment (Fig. 3C), we did observe that 25 μM GM6001 inhibited the MMP-7-associated reduction in NR2A.

In Fig. 3A–C, the antibody used to detect NR2A was raised against amino acids 1099–1213 and would thus not detect an N-terminal fragment. An appropriately sized C-terminal fragment (>80 kDa) was not evident in the MMP-7-treated extracts, suggesting that the C-terminal stub was rapidly internalized and/or degraded, presumably via a mechanism that differs from that which regulates the internalization of full-length receptors. Proteolytic degradation is supported by data shown in Fig. 3B, in which novel low-molecular-mass fragments can be observed only in those lanes in which lysates from MMP-7-treated slice preparations were run. Of note is that proteolytic degradation of a C-terminal fragment between amino acids 516 and 517 and also with the size of fragments seen in previous experiments. C) Schematic representation of NR1 with the cleavage site for MMP-7 (arrow), the ligand binding domain (magenta), and binding sites for the antibodies used in Fig. 1 (Ab1) and in panel A (Ab2).
fragment following ectodomain cleavage of the parent protein has been described and linked to proteolysis by γ secretase (40) and/or the proteosome (41, 42).

Because of the relatively high surface expression of NR2A and the substantial reduction in full-length subunit integrity with MMP-7, we tested the possibility that immunocytochemical analysis would show an appreciable reduction in this subunit following treatment of cells with the proteinase. Results are shown in Fig. 3D, E and, consistent with Western blot results, show reduced NR2A immunofluorescence in MMP-7-treated cells. Because of the likely association of the cleaved NR1 fragment with extracellular matrix, differential processing of the subunit following cleavage, and/or relatively low surface expression, we were not able to demonstrate a similar reduction in NR1 as determined by immunocytochemical analysis (data not shown).

MMP-7 does not cleave AMPA receptor subunits

To test specificity of the MMP-7 effect on NMDA receptor subunits, we also examined AMPA receptor (AMPAR) subunits. Although there is a significant degree of sequence homology between NMDA and AMPA receptor subunits, there are also substantial differences. For example, while NR1 and NR2A have the sequence LTINNE beginning with amino acids 517 and 512, respectively, the gluR1 and gluR2 AMPAR subunits instead have an LTITIV and LTITLV beginning at 493 and 500, respectively. Some of these differ-
ences could be significant in that MMP target sequences may be defined by tertiary structure. Leucine (L), isoleucine (I), and valine (V) are all nonpolar, while asparagine (N) is polar and glutamic acid (E) is both polar and negatively charged.

Shown in Fig. 4 are experiments in which we examined AMPAR subunits gluR1 and gluR2 in control and MMP-7-treated slice lysates. In Fig. 4A, a primary antibody to the N-terminal domain of gluR1 was used to probe the same lysates shown in the 4 left lanes of Fig. 3 which immunoreactivity for full-length NR2A had been reduced by MMP-7. As an additional control, these same lysates were also tested for the presence of the N-terminal NR1 fragment shown in Fig. 1. In contrast to what was observed for NMDAR subunits, we did not observe cleavage products or a diminution of full-length AMPA receptor subunits.

**Pretreatment of dissociated cultures with MMP-7 diminishes NMDA-induced calcium responses**

We next explored the potential for MMP-7 to influence NMDAR function. Cleavage of NMDAR subunits could change receptor conformation, so as to enhance NMDAR-mediated calcium flux, or it could block ligand binding and ligand-stimulated receptor function. Functional studies were also imperative because MMPs might act by more than one mechanism to influence synaptic proteins, and diverse actions might have opposing effects on function. For example, it has been postulated that MMP-9 generates integrin-binding matrix fragments that stimulate changes in NMDA receptor phosphorylation with enhanced NMDA receptor function (24). In addition, in a different experimental system, MMP-7 itself has been linked to tyrosine phosphorylation of NR2B subunits and NMDAR-dependent actin reorganization in synaptic spines (23).

As shown in Fig. 5, we observed that a 30-min pretreatment of cells with MMP-7 resulted in a reduction in NMDA-stimulated calcium flux. This result is in contrast to what has been reported for t-PA, which cleaves NR1, but not NR2A, at an N-terminal site distant from the ligand binding domain (35).

**Pretreatment of slices with MMP-7 increases the AMPA/NMDA current ratio**

We next investigated the possibility that MMP-7 would influence NMDAR-mediated synaptic currents in CA1
pyramidal neurons in acute hippocampus slices. Because it is difficult to directly compare the amplitude of synaptically evoked NMDAR currents between slices, we determined the effects of prolonged MMP-7 administration on the ratio of AMPA to NMDA receptor components of evoked excitatory postsynaptic currents (eEPSCs). To ensure appropriate tissue penetration of the enzyme, we pretreated slices with MMP-7 (100 nM) for 1 h before recording. Because AMPAR- and NMDAR-mediated synaptic responses are kinetically distinguishable, we determined the AMPAR and NMDAR component by measuring the amplitude of the synaptic current at different time points of the eEPSCs recorded at +40 mV (Fig. 6). We found that the AMPA/NMDA ratio of eEPSC was significantly enhanced in slices pretreated with MMP-7 (n=15), compared with control slices (n=12; P<0.05, unpaired Student’s t test). We then recorded AMPAR-mediated miniature EPSCs (mEPSCs) and found that their amplitude was unaltered by prolonged MMP-7 treatment (14.1±1.0 pA; n=10 in control slices, vs. 15.2±0.9 pA; n=9 in MMP-7 treated slices; P=0.4, unpaired Student’s t test). These results, therefore, suggest that the increase in AMPA/NMDA ratio observed following MMP-7 treatment is unlikely to be accounted for by a change in AMPAR function. Rather, these results are best interpreted as suggesting that prolonged administration of MMP-7 leads to a reduction of NMDA-mediated synaptic current in pyramidal neurons of the hippocampus.

DISCUSSION

The NMDAR receptor is a heterotetramer of two NR1 subunits in combination with two regulatory subunits (NR2A-D, NR3A-C). Physiological activation of this receptor is critical to learning and memory, and pathological activation can stimulate neuronal death (43, 44).

In the present study, we have shown that MMP-7, a member of the MMP family known to have a broad substrate range and to be released from resident cells of the CNS, cleaves NR1, an obligate subunit of the NMDA receptor. Moreover, using a recombinant protein construct, we have identified a potential cleavage site in NR1, between proline 516 and leucine 517. Cleavage at this site may impair ligand binding at the catalytic cleft of MMP-7 or MMP-9. Although MMPs can overlap in terms of substrate specificity, the efficiency with which they act on a given target can vary and, for many substrates, a level of specificity does exist. For example, MMP-1 but not MMP-2, MMP-3, MMP-7, MMP-8, or MMP-9 can stimulate calcium flux linked to the cleavage and activation of PAR-1 (17), and MMP-2, but not MMP-7 or MMP-8, can cleave SDF-1α (46). An additional consideration is that MMP-7 lacks a C-terminal hemopexin-like domain. This may render it less susceptible to inhibition by TIMPs and thus better able to retain activity as it permeates slice preparations.

In the present study, we also show that pretreatment of neurons with MMP-7 (50 nM) is linked to a reduction in NMDA-stimulated calcium flux. Consistent with this finding, we observe that MMP-7 pretreatment of hippocampal slices is associated with an increase in the AMPA/NMDA ratio. These results are consistent with a potential disruption of ligand binding ability, which, as mentioned, may depend in part on Pro 516 (36). Of interest, however, is that the effect of MMP-7 on NMDA-stimulated calcium flux differs from that reported for other proteinases. For example, a 15-min pretreatment of neurons with tissue plasminogen activator (t-PA), at a concentration of 20 μg/ml (286 nM), was shown to potentiate NMDA-stimulated calcium flux (35). However, t-PA was shown to cleave NR1 at arginine 260, which is N-terminal to the ligand binding domain (47). Cleavage in this portion of the subunit would thus not be expected to eliminate ligand binding. In addition, changes in NR2A were not observed.

![Figure 6](https://example.com/f6.png)

**Figure 6.** Current traces depicting electrically evoked synaptic responses obtained while voltage clamping CA1 pyramidal neurons at −60 mV (downward deflection) or at +40 mV (upward deflection) in a control slice, and in a slice pretreated for 1 h with 100 nM MMP-7. The AMPA/NMDA ratio was calculated by estimating the respective AMPA and NMDAR contributions on the traces at +40 mV based on their different time course [inset: 1) predominantly AMPA; 2) NMDA component]. The computed AMPA/NMDA ratio was significantly higher (P<0.05, Student’s t test) in slices treated with MMP-7 (n=15), compared to controls (n=12).
One possibility is that t-PA alters NMDAR conformation so that ligand binding or calcium flux is facilitated. The serine protease thrombin has also been shown to potentiate NMDAR function (34). Of interest, however, is that thrombin does so via its ability to stimulate intracellular signaling events through the activation of the G protein-coupled protease activated receptor-1/PAR-1 (34). Thrombin’s potentiation of NMDAR function was diminished by an inhibitor of serine/threonine kinase activity and by the absence of PAR-1.

Changes in the phosphorylation of NMDAR subunits are well known to influence receptor function. Phosphorylation of specific residues may influence factors, including membrane localization, insertion, and deletion/endocytosis (48, 49). While our study is focused on MMP-7-mediated proteolysis of NMDAR subunits, and our calcium and AMPA/NMDA results are consistent with a reduction in ligand binding, we do not rule out the possibility that MMP-7 can influence NMDAR function and/or synaptic plasticity by alternative, non-mutually exclusive mechanisms, including phosphorylation. With respect to this possibility, it is becoming increasingly apparent that when released in a physiologically appropriate manner, select MMPs might enhance neurotransmitter receptor function (50). MMPs can act as signaling molecules through a variety of mechanisms, including interaction with cell surface integrins (16) and the activation of proneurotrophins (51). Nonetheless, while physiological release of select MMPs may enhance synaptic function, our results support that possibility that with chronic inflammation and long-term exposure to high levels of MMP-7, NMDAR subunit cleavage may occur to an extent sufficient to affect an overall reduction in the bioavailability and function of this critical receptor.

In summary, our data show that MMP-7 can cleave NR1, an obligate subunit of the NMDA-type glutamate receptor. Moreover, pretreatment of neurons with MMP-7 is associated with a reduction in NMDA stimulated calcium flux. It is tempting to speculate that proteolysis of NMDAR subunits may be partially protective in conditions characterized by excess protease release in that Ca^{2+} influx through NMDAR contributes to neuronal injury in stroke and age-related neurodegenerative disorders (52–54).

Conditions characterized by excess protease release include CNS inflammation, hypoxia, and excitotoxicity (6, 7, 55–62). It should be noted, however, that long-term loss of NMDARs is likely maladaptive. The NMDA receptor plays a critical role in learning and memory (43), visual plasticity, fear conditioning, and the development of somatotopic maps (44). The induction of long-term potentiation, a potential mechanism for learning, is dependent on NMDAR-mediated calcium flux (44, 63). It has been shown that NR1 is critical for spatial memory and for the preservation of remote memory (64, 65), and one can imagine that a long-term reduction in NMDAR signaling may reduce synaptic stability.

Consistent with this possibility is recent work showing that knockdown of NMDARs leads to a loss of spines and excitatory synapses (66). Future in vivo studies will thus be critical to identify MMP-7-mediated effects that are relevant to normal physiology and pathology.

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