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Differential expression of protease M/neurosin in oligodendrocytes and their progenitors in an animal model of multiple sclerosis

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Abstract

To determine the possible involvement of protease M/neurosin in demyelinating diseases of the CNS, we examined its expression in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), a recognized animal model of multiple sclerosis (MS). In situ hybridization, immunohistochemistry and quantitative real-time polymerase chain reaction (PCR) demonstrated that EAE caused an increase in the expression of protease M/neurosin mRNA and its protein product throughout the white and gray matter surrounding demyelinating lesions. Combined in situ hybridization and immunohistochemistry demonstrated that most of the cells expressing protease M/neurosin mRNA within control spinal cord showed immunoreactivity for CNPase or NG2, cell-specific markers for oligodendrocytes and their progenitors, respectively. In the spinal cord from mice with EAE, the expression of protease M/neurosin mRNA in CNPase-positive cells appeared to be increased while double-labeled cells positive for protease M/neurosin mRNA and NG2 were rarely found in areas associated with demyelinating lesions. Although a prominent accumulation of inflammatory cells including T-cells was observed in the vicinity of demyelinated lesions, these cells were not associated with protease M/neurosin mRNA expression. The levels of protease M/neurosin mRNA expression were unchanged in the spleen and even decreased in the thymus during the course of EAE. These observations suggest that the differential expression of protease M/neurosin in mature oligodendrocytes and their progenitors is involved in the pathogenesis of MS and EAE.

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS) induced by the active or passive immunization of susceptible strains of rodents and primates with myelin components, which is used to study human demyelinating diseases, such as multiple sclerosis (MS) [13, 14]. In C57BL/6 mice, immunization with myelin oligodendrocyte glycoprotein (MOG) induces a progressive disease, which is characterized by demyelination and inflammation in the CNS [9, 10]. Although considerable progress has been made in understanding the genetic susceptibility and pathogenesis of this disease, there is still no uniformly effective treatment strategy [15, 18]. The enzymatic digestion of the blood brain barrier and myelin protein by serine proteases is known to contribute to the development and progression of MS and EAE [1, 12, 19]. The identification and characterization of key enzymatic players may suggest new therapeutic targets.

Kallikreins are serine proteases and known to serve a variety of physiological function [3]. Protease M/neurosin is a kallikrein-like serine protease that is distributed exclusively in oligodendrocytes in the brain [21], and *KLK6* was assigned to a gene in the human genome [8, 22, 24]. Previously, several groups have reported significant reductions of human kallikrein 6 in cerebrospinal fluid and brain extracts of patients with Alzheimer's disease, suggesting that kallikrein 6 may have antiamyloidogenic potential through proteolytic activity [7, 25]. Our recent study has demonstrated that traumatic injury to the spinal cord causes changes in the expression of protease M/neurosin in oligodendrocytes and their progenitors [20]. Scarisbrick et al. [5, 16, 17] demonstrated that expression of protease M/neurosin was upregulated in response to excitotoxic injury to the CNS or demyelinating diseases of the CNS. They also showed that inhibition of the enzymatic activity of protease M/neurosin attenuated the clinical and histological signs

of EAE in myelin proteolipid protein (PLP)-primed SJL/J mice [2]. Although previous studies suggest the possible involvement of protease M/neurosin in the pathogenesis of EAE, changes in the expression of this protease in oligodendrocytes and their progenitor cells during the course of EAE have not been elucidated. In this study we aim to analyze the expression of protease M/neurosin in the CNS of mice with MOG-induced EAE.

The procedure was approved by the Institutional Committee for Experimental animals. C57BL/6 mice were bred in-house (Division of Laboratory Animal Resources at Asahikawa Medical College). Adult (6- to 8-week-old) female mice were used in all experiments (n = 36). MOG_{35-55} peptide (AAs: MEVGWYRSPFSRVVHLYRNGK) was purchased from Sigma-Genosys. Mice were immunized subcutaneously in the four limb flanks with 200 μg of MOG_{35-55} peptide in 100 μl of phosphate-buffered saline (PBS, pH 7.4) and 100 μl of complete Freund's adjuvant (CFA) containing 400 μg of heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) and injected intraperitoneally with 200 ng of pertussis toxin (Sigma, St Louis, MO), on the day of immunization and 2 days later. The clinical signs were scored daily for up to 42 days post-immunization (n = 12) on a scale of 0-5 as follows: 0, no detectable clinical signs; 1, weakness of the tail or abnormal gait; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead.

At days 0, 10, 21 and 42 post-immunization, animals (n = 3 at each time point) were anesthetized with an intraperitoneal injection of pentobarbital sodium (Nembutal, 100 mg/kg) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The spinal cord was removed, postfixed overnight in

the same fixative and then immersed in 30% sucrose in 0.1 M PB for 1-2 days. The spinal cord was then frozen in powdered dry ice, embedded in Tissue-Tek (Miles, Elkhart, IN) optimal cutting temperature compound, and stored at –80 ˚C prior to use. Frozen 14 µm transverse sections through the lumber spinal cord were cut on a cryostat and mounted onto silane-coated slides.

Protease M/neurosin was expressed in 293 cells with His tag at C-terminal. Recombinant protease M/neurosin was purified in a single step utilizing Ni-NTA agarose column. Sample was eluted with 50 mM acetic acid, 100 mM NaCl, pH 4.5. Rabbit polyclonal antibody against protease M/neurosin was generated by immunizing New Zealand white rabbits intradermally with 250 µg of active recombinant protease M/neurosin in Freund's complete adjuvant, followed by three boosts with 125 µg every two weeks with Freund's incomplete adjuvant. The IgG fraction was purified over a protein A column. The purified IgG fraction was further purified by protease M/neurosin-affinity column, and eluted with 20 mM acetic acid, 150 mM NaCl, pH 3.0. Then adjusted pH to 7.3 with 1 M Tris-HCl, pH 7.5.

Immunohistochemistry was performed using rabbit anti-protease M/neurosin antibody as described above. The method used for immunohistochemistry was described in previous papers [20, 23]. In situ hybridization was performed using a digoxigenin (DIG, Roche Molecular Biochemicals, Mannheim, Germany)-labeled cRNA probe for protease M/neurosin mRNA. The method used for in situ hybridization was described in previous papers [4, 20, 23]. Some sections were further processed for immunolabeling with the following cell-specific markers: mouse monoclonal anti-glial fibrillary acidic protein (GFAP, Sigma; 1:5,000) to identify astrocytes; mouse monoclonal anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, Sigma; 1:2,000) to identify mature

oligodendrocytes; mouse monoclonal anti-microtubule-associated protein 2 (MAP2, Sigma; 1:1,000) to identify neurons; rat monoclonal anti-F4/80 (BMA Biomedical AG, Augst, Switzerland; 1:500) to identify macrophages and microglial cells; rabbit polyclonal anti-NG2 (Chemicon, Temecula, CA; 1:500) to identify oligodendrocyte progenitors; rat monoclonal anti-CD4 (Serotec, Oxford, UK; 1:50) or CD8b (Serotec; 1:50) to identify T-cells; and rat monoclonal anti-CD45 (Serotec, Oxford, UK; 1:500) to identify all leukocytes. Alexa-568 and Alexa-594 (Molecular Probes, Eugene, OR)-conjugated secondary antibodies were used to visualize primary antibody binding (red signals). The number of cells positive for protease M/neurosin mRNA (dark brown), and either CNPase or NG2 (red), was quantified by counting all single- or double-labeled cells at a magnification of 40×. More than 1,000 cells were identified in the lumber spinal cord of three different animals. The number of cells was determined and expressed as a percentage of the total number of cells labeled.

Quantitative real-time polymerase chain reaction (PCR) was performed using the LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics, Penzberg, Germany). At different time points during the course of EAE, mice (n = 3 at each time point) were given a lethal dose of anesthetic (100 mg/kg), and the spinal cord, spleen and thymus were quickly dissected. The methods used for RNA extraction and PCR was described in a previous paper [20]. The PCR primers were designed according to published sequences of protease M/neurosin, CCCAGATACCATTCAGTGT (sense); CGTGGGGGAGAACTGGATGT (antisense), tumor necrosis factor-α (TNF-α), AATTCACTGGAGCCTCGAATG (sense); CCCGGCCTTCCAAATAAAT (antisense), and glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal control, CGGGAAGCCCATCACCATCA (sense); GAGGGGCCATCCACAGTCTT (antisense).

The raw data from three individual experiments were used for statistical analysis (analysis of variance [ANOVA] with the post hoc Fisher's protected least significant differences [PLSD] test).

All animals exhibited signs of disease and developed a chronic course (Fig. 1A). The PCR analysis demonstrated that the expression of mRNA encoding protease M/neurosin was upregulated during the course of EAE. Protease M/neurosin mRNA expression showed a significant increase at day 21 and persisted through to day 42 post-immunization (Fig. 1B). To estimate inflammatory loci, spinal cord sections from mice with EAE at day 21 post-immunization (grade 4) were processed for Luxol fast blue/cresyl violet (LFB/CV) histological staining. As shown in Fig. 2A, there were multiple foci of obvious defects in LFB staining (indicating demyelination) and inflammatory cell infiltrates in the spinal cord. In situ hybridization and immunohistochemistry demonstrated an increase in the expression of protease M/neurosin mRNA and its protein product in areas associated with these demyelinating foci (Fig. 2B-F). Within untreated control mice, constitutive expression of protease M/neurosin mRNA was observed in the white matter of the spinal cord (Fig. 2B). In mice with EAE, enhanced expression of protease M/neurosin mRNA was observed throughout the white and gray matter (Fig. 2C, D). Similarly, immunohistochemistry showed constitutive expression of protease M/neurosin within control spinal cord white matter and enhanced expression in areas associated with demyelinating foci (Fig. 2E, F). At day 42 post-immunization, when there was some attenuation of the clinical symptoms and inflammatory pathology, the increased expression of protease M/neurosin still persisted (data not shown).

To determine the identity of the cells expressing protease M/neurosin mRNA in the

spinal cord with demyelinating lesions, a combination of in situ hybridization and immunohistochemistry was performed in the same spinal cord sections from control mice and mice with EAE. The vast majority of the cells (93.1%, 507/544 cells) expressing protease M/neurosin mRNA were immunoreactive for CNPase in the spinal cord white matter from control mice (Fig. 3A). In addition, a relatively small fraction of the cells (42.4%, 372/877 cells) expressing protease M/neurosin mRNA showed NG2-immunoreactivity (Fig. 3B). Protease M/neurosin mRNA labeling did not colocalize with immunoreactivity for GFAP, MAP-2 or F4/80 (data not shown). Thus, a subpopulation of CNPase- or NG2-immunoreactive cells expressed protease M/neurosin mRNA within control spinal cord. After the induction of EAE, the composition of the protease M/neurosin mRNA-expressing cell population was altered. Although the number of double-labeled cells positive for protease M/neurosin mRNA and CNPase appeared to be increased in the spinal cord with demyelinating lesions (Fig. 3C), a subset of the cells (25.8%, 210/811 cells) expressing protease M/neurosin mRNA did not colocalize with CNPase (Fig. 3C, arrowheads). Interestingly, double-labeled cells positive for protease M/neurosin mRNA and NG2 were rarely observed (4.2%, 39/929 cells) in areas associated with demyelinating lesions (Fig. 3D). These results suggests that protease M/neurosin mRNA expression in oligodendrocyte progenitors was decreased, while the expression in CNPase-positive oligodendrocytes appeared to be increased during the course of EAE. Protease M/neurosin mRNA labeling did not colocalize with immunoreactivity for GFAP, MAP-2 or F4/80 in the spinal cord of mice with EAE (Fig. 3E). Although a prominent accumulation of CD4- and CD8-positive T-cells as well as CD45-positive leukocytes was observed in areas associated with demyelinating foci, these cells were not associated with protease M/neurosin mRNA labeling (Fig. 3F).

Quantitative real-time PCR analysis in the spleen demonstrated that there were no significant changes in the expression of protease M/neurosin and TNF-α mRNAs during the course of EAE (Fig. 4A, B). In the thymus, however, protease M/neurosin mRNA expression was significantly downregulated at days 10 and 21 post-immunization while TNF-α mRNA expression was upregulated at day 21 post-immunization (Fig. 4C, D).

This study shows an increase in the expression of protease M/neurosin mRNA and its protein product in an animal model of MS, suggesting the possible involvement of this protease in the pathogenesis of demyelinating diseases of the CNS. Within control spinal cord, protease M/neurosin mRNA was expressed in CNPase- or NG2-positive cells, suggesting that protease M/neurosin has some role in immature as well as mature oligodendrocytes under normal conditions. Alternatively, a subpopulation of protease M/neurosin mRNA-expressing cells could be oligodendrocytes of intermediate maturity, which are positive for both CNPase and NG2. Cells expressing both NG2 and CNPase were reported in the rat CNS [6]. In the spinal cord from mice with EAE, the expression of protease M/neurosin within a subset of CNPase-positive cells appeared to be increased while the expression within NG2-positive oligodendrocyte progenitors was decreased. It is possible that oligodendrocyte progenitors, which express protease M/neurosin, differentiate into CNPase-positive mature oligodendrocytes and are involved in the pathogenesis of EAE. Alternatively, oligodendrocyte progenitors, which do not express protease M/neurosin, divide at or migrate to demyelinating sites. As a result of increase in the total number of oligodendrocyte progenitors, the percentage of double-labeled cells positive for protease M/neurosin and NG2 might be decreased. Previous studies have demonstrated that the number of NG2-positive oligodendrocyte progenitors increased in

response to demyelination and decreased after the onset of remyelination [6, 11].

Previous study has demonstrated that CD4- or CD8-positive T-cells express protease M/neurosin in the spinal cord of mice with Theiler's murine encephalomyelitis virus-induced EAE [16]. In the present study, however, the prominent infiltration of inflammatory cells was observed in the vicinity of demyelinated lesions but these cells were not associated with protease M/neurosin mRNA expression. A difference in inflammatory responses between MOG-induced and Theiler's murine encephalomyelitis virus-induced EAE models could account for the difference in the expression of protease M/neurosin in inflammatory cells. Indeed, differential T- and B-cell responses have been reported depending on the encephalitogenic component such as myelin basic protein, myelin-associated glycoprotein, PLP or MOG and depending on the animal species [9]. In the present study we also showed that levels of protease M/neurosin mRNA expression were unchanged in the spleen and even decreased in the thymus during the course of EAE. Although the identity of the cells expressing protease M/neurosin mRNA in the spleen and thymus remains to be determined, it is likely that leukocytes including lymphocytes are not a major source of protease M/neurosin in MOG-induced EAE.

In conclusion, changes in the expression of protease M/neurosin in oligodendrocytes, their progenitors or other cell types may be involved in the pathogenesis of demyelinating diseases of the CNS.

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Figure legends

Figure 1

Clinical course and changes in the expression of protease M/neurosin mRNA during the course of MOG-induced EAE in C57BL/6 mice. A, The disease severity was scored daily on a clinical scale from 0 to 5 and the mean score (± SEM) for each day was calculated. B, Levels of specific mRNA were measured by real-time PCR and normalized with the level of GAPDH mRNA expression in each individual sample. Each bar represents the mean ± SEM of 3 individual experiments. Statistical comparisons were made among all groups using raw data. Asterisks indicate a significant difference from naive control (ANOVA with post hoc Fisher's PLSD test), $*P < 0.05$.

Figure 2

Expression of protease M/neurosin mRNA and its protein product in the spinal cord of mice with EAE (grade 4). A, Frozen cross-sections of spinal cord of mice with MOG-induced EAE were stained with LFB and CV. Note that extensive demyelination and infiltration of inflammatory cells were evident in the spinal cord at day 21 post-immunization (arrows). In situ hybridization was performed with a probe for protease M/neurosin mRNA (B-D) in sections of spinal cord from control mice (B) and mice with MOG-induced EAE (C,D). B, Within untreated control mouse spinal cord, constitutive expression of protease M/neurosin mRNA was observed in the white matter. Inset in B, higher magnification of the boxed area. C, Enhanced expression of protease M/neurosin mRNA was observed throughout the white and gray matter surrounding demyelinating lesions. D, Higher magnification of the boxed area in C.

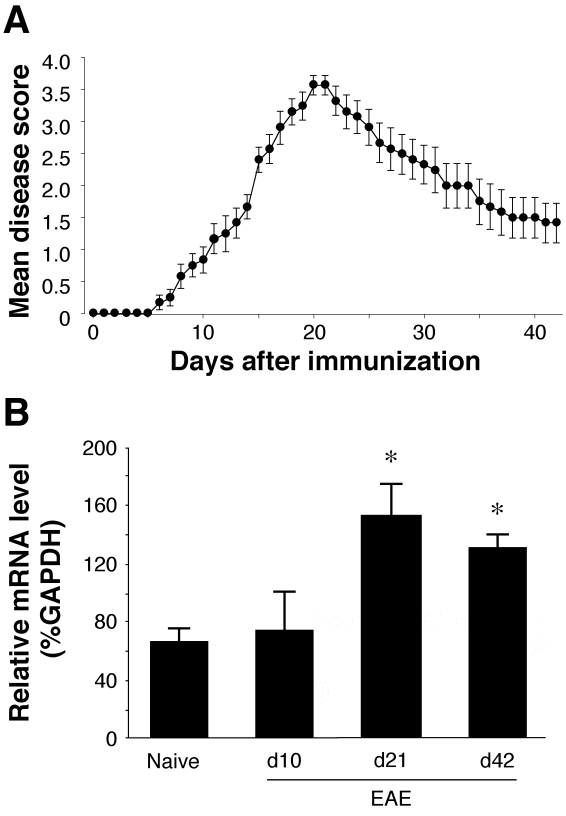
Immunohistochemistry was performed with a polyclonal antibody specific for protease M/neurosin in sections of spinal cord from control mice (E) and mice with MOG-induced EAE (F). Scale bars: F, 500 μm for A-C, 100 μm for D-F, inset in B, 200 μm.

Figure 3

Combined in situ hybridization for protease M/neurosin mRNA (dark brown) and immunohistochemistry for cell-specific markers (red) in the spinal cord from control mice (A,B) and mice with EAE (C-F). Double-labeled cells (arrows) and single-labeled cells positive for protease M/neurosin mRNA (arrowheads) are shown. Within control spinal cord, most of the cells expressing protease M/neurosin mRNA showed immunoreactivity for CNPase (A) or NG2 (B). C, The number of double-labeled cells positive for protease M/neurosin mRNA and CNPase was increased in the spinal cord of mice with EAE (arrows), but single-labeled cells positive for protease M/neurosin mRNA were also found (arrowhead). D, Double-labeled cells positive for protease M/neurosin and NG2 were rarely found in areas associated with demyelinating lesions. Protease M/neurosin mRNA-expressing cells did not show immunoreactivity for GFAP (E) or CD45 (F). Scale bar: 50 μm.

Figure 4

Changes in the expression of protease M/neurosin and TNF-α mRNAs in the spleen (A,B) and thymus (C,D) during the course of EAE. Each bar represents the mean ± SEM of 3 individual experiments. Statistical comparisons were made among all groups using raw data. Asterisks indicate a significant difference from naive control (ANOVA with post hoc Fisher's PLSD test), $*P < 0.05$; $***P < 0.001$.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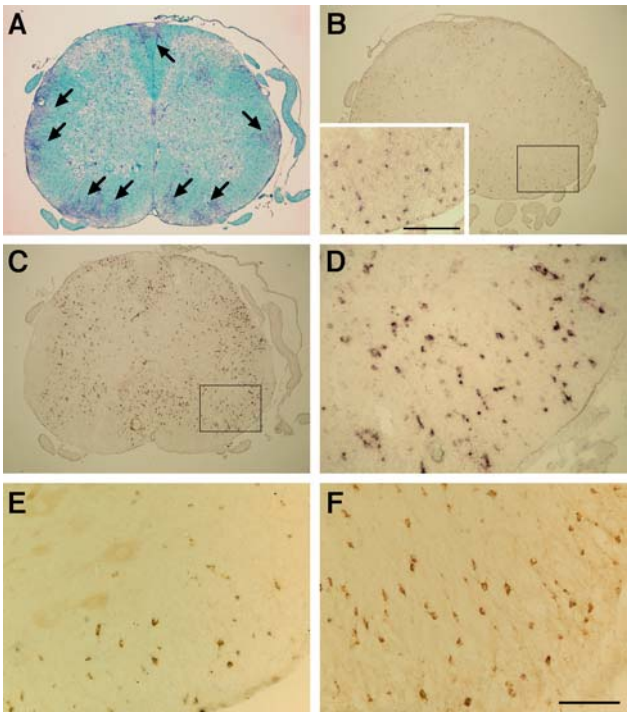
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
Days after immunization
B
Relative mRNA level (%GAPDH)
200
160
120
80
40
0
*
*
Naive
d10
d21
d42
EAE

Fig. 1


A
B
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E
F

Fig. 2

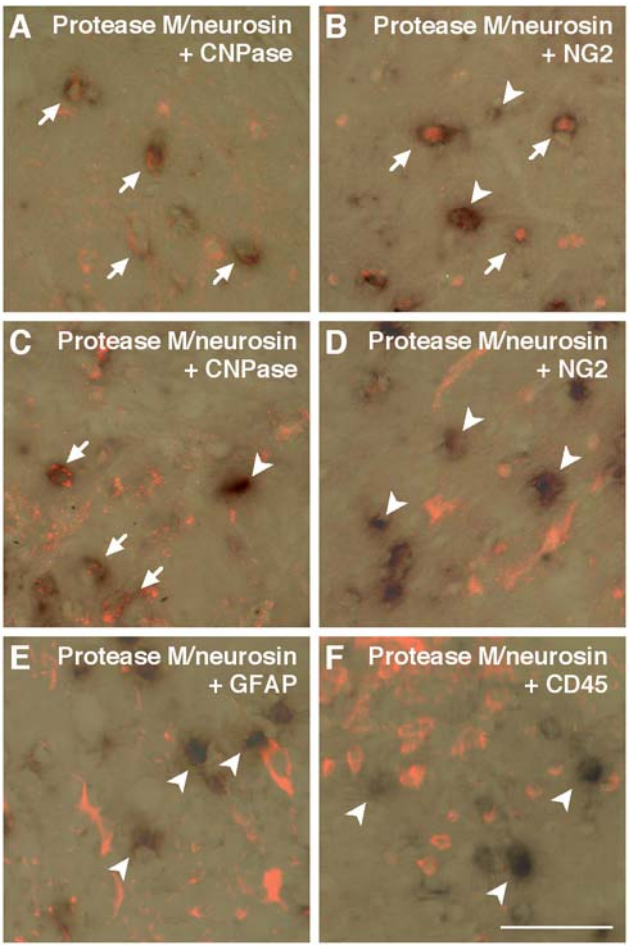
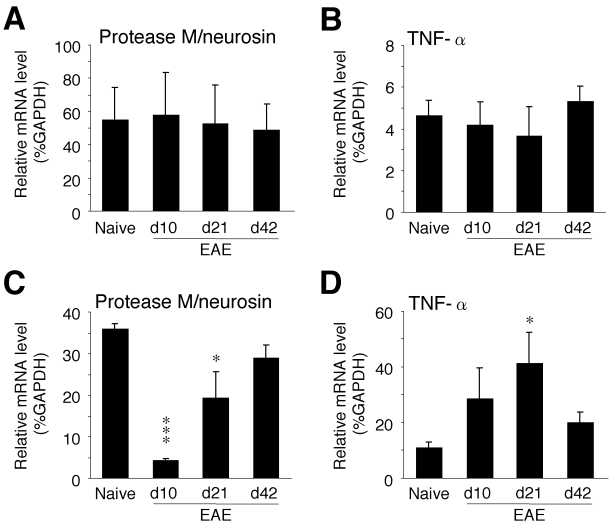

A Protease M/neurosin + CNPase
B Protease M/neurosin + NG2
C Protease M/neurosin + CNPase
D Protease M/neurosin + NG2
E Protease M/neurosin + GFAP
F Protease M/neurosin + CD45

Fig. 3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Protease M/neurosin
Relative mRNA level (%GAPDH)
100
80
60
40
20
0
Naive
d10
d21
d42
EAE
B
TNF- α
Relative mRNA level (%GAPDH)
8
6
4
2
0
Naive
d10
d21
d42
EAE
C
Protease M/neurosin
Relative mRNA level (%GAPDH)
40
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Relative mRNA level (%GAPDH)
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EAE

Fig. 4