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Enhanced expression of matrix metalloproteinase-12 contributes to Npc1 deficiency-induced axonal degeneration

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Abstract

Niemann-Pick type C (NPC) disease is a genetic disorder associated with intracellular cholesterol accumulation in brain and other organs, and neurodegeneration is generally believed to be the fatal cause of the disease. In view of the emerging role of matrix metalloproteinase-12 (MMP-12) in neuronal injury, we investigated its expression and potential roles in axonal degeneration in Npc1^{-/-} mouse brain. Microarray and quantitative real-time reversed transcription PCR analysis indicated a marked increase in MMP-12 mRNA levels in cerebellum of 3 week-old Npc1^{-/-} mice, as compared to wild-type littermates. Western blots showed that the ratio of mature MMP-12 over pro-MMP-12 was significantly increased in cerebellum of Npc1^{-/-}, as compared to wild-type mice. Immunohistochemical studies confirmed that MMP-12 expression was increased, especially in the cell bodies of Purkinje neurons in Npc1^{-/-} mice. Neuritic growth was significantly reduced by Npc1 siRNA knockdown in nerve growth factor-differentiated PC-12 cells, and this effect was completely reversed by treatment with an MMP-12 specific inhibitor. Furthermore, in vivo experiments showed that chronic treatment with the MMP-12 inhibitor ameliorated Npc1 deficiency-induced axonal pathology in the striatum. Our results indicate that abnormal neuronal expression of MMP-12 may contribute to axonal degeneration in NPC disease, thus providing a potential novel target for treatment.

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Competing interests

No competing interest

Authors' contributions

Conceived and designed the experiments: GL, MB and XB. Performed the experiments: GL, ZW, SYM, EL, and OA. Analyzed the data: GL, ZW, EL and XB. Wrote the paper: GL, MB and XB.

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Keywords

axonal degeneration; EMMPRIN; MMP-9; MMP-12; myelination; Niemann-Pick type C; siRNA knockdown

Background

Niemann-Pick type C disease (NPC) is a rare lysosomal storage disorder caused by genetic mutations in either *NPC1* (95% of patients) (Carstea *et al.*, 1997) or *NPC2* (5% of patients) (Naureckiene *et al.*, 2000) genes. The pathology is characterized by accumulation of cholesterol and other lipids in endosomes-lysosomes in multiple organs, including liver and brain (Rosenbaum and Maxfield, 2011; Vance and Peake, 2011). Intriguingly, although NPC proteins are ubiquitously distributed throughout the body (Kwon *et al.*, 2009), the most prominent pathological feature of the disease is progressive neuronal death, particularly of neurons in cerebellum, cortex, thalamus and brainstem [reviewed in (Walkley and Suzuki, 2004)]. Previous studies have shown that neurodegeneration in animal models of NPC is initiated in axons, and progresses to cell bodies (March *et al.*, 1997; Ong *et al.*, 2001; Sarna *et al.*, 2003). In addition, glial activation has also been proposed to contribute to neurodegeneration (Baudry *et al.*, 2003; German *et al.*, 2002).

Matrix metalloproteinases (MMPs) are secreted proteins and several of them are expressed in the central nervous system (CNS). MMPs degrade extracellular matrix components and cell-surface proteins, and have been proposed to play important roles in normal brain function and in various neurological disorders (Sbai *et al.*, 2008; Ethell and Ethell, 2007; Ulrich *et al.*, 2005; Milward *et al.*, 2007). For instance, MMP-2 is expressed in neurons and participates in synaptic remodeling (Fredrich and Illing, 2010), axonal regeneration and astrocyte migration after injury (Hsu *et al.*, 2006). MMP-9 is enriched in adult brain and has been reported to be critical for the induction of long-term potentiation and for learning and memory (Bozdagi *et al.*, 2007). Along this line, it has been reported that TIMP-1, an endogenous MMP inhibitor, also plays important roles in learning and memory in mice (Chaillan *et al.*, 2006). Although MMP-9 deficiency affects axonal outgrowth in cerebellum (Vaillant *et al.*, 2003), microinjection of activated MMP-9 and MMP-2 into subcortical white matter causes severe axonal injury (Newman *et al.*, 2001), indicating the importance of maintaining a critical level of these MMPs for normal brain function.

MMP-12 has been shown to play important roles in microglial activation (Power *et al.*, 2003; Vos *et al.*, 2003), oligodendrocyte maturation and myelination (Larsen and Yong, 2004). Results from in vitro experiments indicate that MMP-12 degrades myelin basic protein (MBP), a protein expressed by oligodendrocytes (Chandler *et al.*, 1996; Gronski *et al.*, 1997). Abnormal expression of MMP-12 has been linked to some central nervous system diseases. For example, MMP-12 expression in microglia is up-regulated by A β 1–42 application (Ito *et al.*, 2007). Likewise, levels of MMP-12, MMP-3, TIMP-1, are markedly increased in a mouse model of multiple sclerosis (MS), an autoimmune disease characterized by demyelination and axonal degeneration (Ulrich *et al.*, 2006). MMP-12 has also been found in phagocytotic macrophages in lesion sites of MS patient brains (Vos *et al.*, 2003). Since axonal degeneration and hypomyelination are among the earliest onset

pathological features of NPC, we investigated the potential involvement of MMP-12 in axonal injury in brain of *Npc1*-deficient (*Npc1*^{−/−}) mice.

Materials and Methods

Animals

Npc1^{NIH} (BALB/cNctr-*Npc1*m1N/J) heterozygous mice on BALB/c background were purchased from Jackson Laboratory (Bar Harbor, ME) and *Npc1*^{−/−} mice were produced by in-house breeding from heterozygous mice. *Npc1* genotype was determined with PCR method as previously described (Baudry *et al.*, 2003). Animals were housed under controlled temperature on a 12/12-h light/dark cycle and given access to water and a standard rodent diet ad libitum. The use of animals was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and animal husbandry, care and experimental protocols were approved by the local Institutional Animal Care and Use Committee.

Quantitative real-time PCR

Total RNA (1–2 µg) was reversed transcribed into cDNA with oligo (dT) using SuperScript III first-strand synthesis system according to the manufacturer's instruction (Invitrogen). cDNA was amplified by primer pairs specific for MMP-12 (5'-TTT CTT CCA TAT GGC CAA GC-3' forward, 5'-GGT CAA AGA CAG CTG CAT CA-3'reverse). GAPDH primers (5'-AAC TTT GGC ATT GTG GAA GG-3'forward, 5'-ACA CAT TGG GGG TAG GAA CA-3'reverse) were used as internal control. Quantitative real-time PCR was done with Applied Biosystem 7300 real-time PCR system (Foster City, CA) using SYBR-Green. Each sample was analyzed in triplicate and the relative quantification of gene expression was performed with the comparative cycle number measured with the threshold (C_T) method (Liao *et al.*, 2010).

Drug treatment

MMP-12-inhibitor (MMP408, Millipore, Billerica, MA) was dissolved in a vehicle solution of 50% DMSO to make a stock solution. At postnatal day (P) 7–9, *Npc1*^{−/−} and wild-type mice were randomly assigned to either vehicle or MMP-12 inhibitor treatment group and given daily subcutaneous injections for 21–23 days.

Immunohistochemistry

Mice were sacrificed by deep anesthesia with pentobarbital, and perfused with 4% paraformaldehyde (PFA). Brains were cut into 30 µm coronal or sagittal sections for forebrain and cerebellum, respectively. For immunohistochemistry, floating tissue sections were washed in 0.1M phosphate buffer (PB), blocked with 10% horse serum and 4% goat serum; sections were then incubated with primary antibodies in blocking buffer overnight at 4 °C. Sections were then washed with PB before being incubated with secondary antibodies (conjugated with Alexa-594 or Alexa-488). After four more washes, sections were then mounted onto Superfrost® plus slides (VWR, West Chester, PA). Images were taken by a blind observer using the Nikon EZ-C1 software ver. 2.10; the same acquisition parameters were used across different experimental groups and images were analyzed with ImageJ

software. Antibodies used were: MMP-12 antibody (1:25, Santa Cruz Technology, Santa Cruz, CA or 1:1000 Abcam, Cambridge, MA), EMMPRIN antibody (1:200, Abcam, Cambridge, MA), CNPase antibody (1:250, Millipore, Temecula, CA), SMI-312 (1:1000, Convance, San Diego, CA), and MBP (1:500, Santa Cruz). Quantification of MMP-12 immunoreactivity in the cerebellar cortex was done with images taken with a 100× objective from the same region in the same lobe among different experimental animals, and 3 mice per group were used. Quantification of neurofilament (SMI-312)-ir areas of fasciculated bundles in the striatum was performed on images of the caudoputamen taken with a 20× objective at the same coronal level from different mice. Means of SMI-312-ir areas were first obtained from at least 2–4 sections per mouse and group means were then calculated from 3–7 different mice for each experimental group and were expressed as percentage of values from wild-type mice.

Western blots

Mice were decapitated under deep anesthesia and tissues from different brain regions (cortex, hippocampus, cerebellum, brainstem) were collected. Whole homogenates were prepared in aCSF (124 mM NaCl, 3 mM KCl, 1.25 mM KH₂PO₄, 2.5 mM MgSO₄, 3.4 mM CaCl₂, 26 mM NaHCO₃, 10 mM Glucose, protease inhibitor cocktail, and phosphatase inhibitor cocktails 1 and 2 from Sigma). Total proteins (10 µg) were separated on 8–10% SDS gels and transferred onto PVDF membranes. Membranes were incubated with primary antibodies in 1% bovine serum albumin followed by incubation with peroxidase-conjugated secondary antibodies (1:10,000; Amersham Biosciences, Piscataway, NJ). Peroxidase reaction was developed with the ECL-plus detection kit. The following primary antibodies were used: Anti-MMP-12 antibody (1:10000, Santa Cruz Biotechnology, CA), and anti-GAPDH antibody (1:30,000, Millipore) was used as loading control. Images were analyzed with ImageJ software. For all experiments, samples from wild-type and *Npc1*^{−/−} mice were loaded onto the sample gels and protein levels were first normalized against GAPDH before any comparison analysis.

PC-12 cell experiments

PC-12 cells (CRL1721) were purchased from ATCC and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium supplemented with 10% horse serum (Sigma, St. Louis, MI) and 5% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were seeded in Poly-L-lysine (P1524, Sigma) coating glass bottom microwell dishes (MatTek Corporation, Ashland, MA) for two days before differentiation with nerve growth factor (300 ng/ml, Sigma) for 10 days. *Npc1* siRNA (Strauss *et al.*, 2010) (100 pmol, Qiagen, Valencia, CA) and scrambled control siRNA were transfected together with a GFP plasmid (2 µg) immediately before differentiation using the X-tremeGENE-siRNA transfection kit according to the manufacturer instructions (Roche). MMP-12 inhibitor (MMP408, 500 nM, Abcam) (Li *et al.*, 2009a; Li *et al.*, 2009b) was added 4 h after transfection. Nine days after transfection, cells were fixed with 2% PFA for 15 min; cells were then examined and imaged using a fluorescence microscope (Nikon Eclipse TE2000-S) and a DS camera from Nikon. Neuritic length was measured as previously described using NIH ImageJ software (Das *et al.*, 2004). Data were expressed as means of four sets of

individual experiments; for each experiment at least 25–26 cells per group were analyzed and averaged values were used to calculate group means.

Statistical analysis

Data are presented as means $\pm$ S.E.M. For experiments where only two groups were compared, two-tail t-test was used for determining statistical significance. When more than 2 groups were compared, we used one-way ANOVA or two-way ANOVA followed by Tukey's post-hoc test to determine statistical significance. P values less than 0.05 were considered statistically significant.

Results

Increased levels of MMP-12 mRNA and active form protein in cerebellum of *Npc1*^{−/−} mice

Using two microarray platforms from Agilent and Illumina, we previously reported that gene expression in six signaling pathways was altered in the cerebellum of 3 week-old *Npc1*^{−/−} mice (Liao *et al.*, 2010). From the same microarray data, we found that MMP-12 mRNA level was increased by 4.7–5.7 fold in *Npc1*^{−/−} mice, while other MMP mRNAs were either unchanged or decreased, as compared to wild-type littermates (Table 1A & B). Real-time reverse-transcribed PCR analysis confirmed that MMP-12 mRNA levels were markedly increased in *Npc1*^{−/−} mice (fold change 142 ± 9 , $p < 0.01$, $n=4$). As cerebellum is the most severely affected structure and exhibits the earliest onset of axonal degeneration in NPC (Ong *et al.*, 2001), these results suggested a potential role of MMP-12 in NPC pathogenesis.

To determine whether increased MMP-12 transcripts resulted in increased protein levels, we analyzed levels of MMP-12 protein in whole homogenates prepared from cerebellum of 3 week-old mice by western blots (Fig. 1). MMP-12 is secreted as an inactive (pro-) form, which becomes active by removal of the pro-peptide sequence. Western blot probed with an antibody recognizing both the inactive and active forms showed that levels of pro-MMP-12 (54 kDa) were significantly decreased ($65 \pm 3\%$, $p < 0.05$, $n=3$) in *Npc1*^{−/−} mice, as compared to wild-type littermates (Fig. 1). Levels of the active form (45 kDa) were not significantly altered, but the ratio of active MMP-12 over pro-MMP-12 was significantly increased ($152 \pm 13\%$, $p < 0.05$, $n=3$). These results suggested that MMP-12 activity was increased in *Npc1*^{−/−} mice, due to increased conversion to the active form.

Altered MMP-12 expression in cortex and cerebellum of *Npc1*^{−/−} mice

To determine MMP-12 cellular expression in *Npc1*^{−/−} mouse brain, we performed immunohistochemistry with two different anti-MMP-12 antibodies (from Santa Cruz Biotechnology and Abcam) in brain sections from mice during early postnatal development. At P15, high levels of MMP-12 immunoreactive (ir) products were observed in apical dendrites of cortical neurons, while moderate levels were found in cell bodies throughout the cortex in wild-type mice (Fig. 2A). By P30, MMP-12-ir was further increased in dendrites and cell bodies of cortical neurons in wild-type mice, as compared to P15 (Figure 2A). Levels of MMP-12-ir in cortical neurons from *Npc1*^{−/−} mice were higher than in wild-type mice, especially at P15, although the staining pattern did not significantly differ from that of

wild-type mice (Fig. 2A). In cerebellum, intense MMP-12-ir was evident in the Purkinje cell layer at P15. High magnification examination revealed that MMP-12-ir was mainly present in cell bodies of Purkinje neurons, especially in the nucleus (Fig. 2B). Quantitative analysis indicated that levels of MMP-12-ir were significantly increased in *Npc1*^{-/-} mice ($179 \pm 15\%$, $p < 0.01$, $n=3$), as compared to wild-type mice. By P30, levels of MMP-12-ir were significantly reduced in cerebellum of wild-type mice, as compared to those at P15, but remained elevated in mutant mice. Levels of MMP-12-ir in cerebellar white matter in *Npc1*^{-/-} mice were comparable or slightly reduced in comparison to wild-type mice (supplemental Fig. 1). In the caudate putamen, MMP-12-ir was mostly found in axon bundles that were also labeled by the SMI-312 antibody (Fig. 3), a pan-marker of neurofilaments. Moderate intensity of MMP-12-ir was occasionally found in small cells immunopositive for CNPase, a marker for oligodendrocytes (data not shown). High MMP-12-ir levels were observed at P7 (Fig. 3A), and gradually decreased from P15 to P30 (Fig. 3B), but no obvious difference was observed at any age between the two genotypes. Quantitative analysis of SMI-312-ir axon bundles revealed a trend of reduction in bundle areas in the caudate putamen of *Npc1*^{-/-} mice, which started at P7 ($69 \pm 9\%$ of wild-type values in *Npc1*^{-/-} mice, $n=3$, $p=0.17$), was maintained at P15 ($74 \pm 6\%$ of wild-type values in *Npc1*^{-/-} mice; $n=6$, $p=0.06$) and the decrease reached statistical significance at P30 ($49 \pm 4\%$ of wild-type values in *Npc1*^{-/-} mice; $n=3$, $p=0.03$; Fig. 3C), which is in agreement with our previous study (Qin *et al.*, 2010),

Altered EMMPRIN expression in cerebellum of *Npc1*^{-/-} mice

Expression of the extracellular matrix metalloproteinase inducer (EMMPRIN, a.k.a. CD147) was also evaluated by immunofluorescence. In cerebellum of P15 wild-type mice, small EMMPRIN-ir puncta were present in cell bodies of Purkinje neurons. EMMPRIN expression was elevated in Purkinje cell bodies in *Npc1*^{-/-} mice (Fig. 4A). Of notice, MMP-12 and EMMPRIN were co-localized in Purkinje neurons in *Npc1*^{-/-} mice at P15 (Fig. 4B). EMMPRIN expression decreased in both genotypes from P15 to P30 (Fig. 4A). EMMPRIN protein levels in the whole cerebellum were not significantly different from those in wild-type mice, as analyzed by western blot (data not shown).

Since MMP-9 mRNA levels were down-regulated in *Npc1*^{-/-} mouse cerebellum in the microarray study (Table 1B), we performed immunohistochemistry with an anti-MMP-9 antibody in brain tissues from *Npc1*^{-/-} mice. Consistent with a previous report (Duran-Vilaregut *et al.*, 2010), MMP-9 was expressed in neurons in cortex and striatum of wild-type mice (data not shown). High levels of MMP-9-ir were also observed in cell bodies and dendrites of Purkinje neurons of both wild-type and *Npc1*^{-/-} mice, and no significant difference was observed between the two genotypes (supplemental Fig. 2).

Npc1 siRNA knockdown reduced neuritic growth in neuron-like cells, an effect reversed by MMP-12 inhibition

To directly test the potential involvement of MMP-12 in *Npc1* deficiency-induced axonal pathology, we used PC12 cells previously differentiated by treatment with nerve growth factor to generate neuron-like cells, with clearly defined neuritic processes. These cells were then transfected with *Npc1* siRNA to generate *Npc1*-deficient neuron-like cells. Image

analysis indicated that neuritic length was significantly shorter in cells treated with Npc1 siRNA, as compared to a scrambled control siRNA (Fig. 5A&B, $62.1 \pm 1.4\%$ of control, $**p < 0.01$; $n=4$ individual experiments). Chronic treatment of Npc1-deficient neuron-like cells with an MMP-12 specific inhibitor, MMP408 (500 nM; see Materials and Methods for treatment details), restored neuritic length to control values (average length of neurites, $112 \pm 9\%$ of control cells; Fig. 5A&B, $\#p < 0.05$, as compared to siRNA treatment alone; $n=4$).

Axonal degeneration in Npc1^{-/-} mice was ameliorated by chronic MMP-12 inhibition

We next tested whether MMP-12 inhibition would also mitigate axonal degeneration in vivo. Npc1^{-/-} and wild-type littermates were injected daily with the MMP-12 inhibitor, MMP408 (5 mg/kg), for 21–23 days starting at P7–9. This inhibitor has been shown to effectively suppress MMP-12-induced lung inflammation and macrophage infiltration and inflammation in retina (Li *et al.*, 2012; Li *et al.*, 2009b). We used the clearly defined reduction in the size of SMI-312-ir axon bundles in the caudate putamen region as the read-out for treatment efficacy. Chronic treatment with MMP-12 inhibitor significantly increased SMI-312-ir in axon bundles in Npc1^{-/-} mice (Fig. 6A), and quantitative analysis showed that SMI-312-ir area in treated Npc1^{-/-} mice was comparable to that in wild-type mice (Fig. 6B). Similarly, MMP-12 inhibitor treatment also increased the area labeled by an antibody against MBP, a myelin marker (not shown). Chronic MMP-12 inhibition did not significantly alter axon bundle size or myelination in wild-type mice.

Discussion

Axonal pathology, including swollen axon hillocks, axonal spheroids, and focal axonal swelling (Sarna *et al.*, 2003; Zervas *et al.*, 2001; Higashi *et al.*, 1993), has been observed in Npc1^{-/-} mice as early as postnatal day 9 (Ong *et al.*, 2001). We also previously showed that early onset axonal degeneration in Npc1^{-/-} mice was associated with loss of p53-mediated suppression of Rho kinase (Qin *et al.*, 2009), resulting in actin depolymerization, disruption of the cytoskeletal network and growth cone collapse (Qin *et al.*, 2010). In the current study, we provided evidence that overactivation of the secreted matrix metalloproteinase, MMP-12, which degrades a variety of proteins, including elastin, collagen type IV, fibronectin, laminin, gelatin, vitronectin, plasminogen, fibrinogen and other matrix proteins (Chandler *et al.*, 1996; Gronski *et al.*, 1997; Hiller *et al.*, 2000), might also contribute to axonal degeneration.

MMP-12 was first identified as a protein secreted by inflammatory macrophages (Banda and Werb, 1981), and has since been found in several cell types, including human airway smooth muscle cells (Xie *et al.*, 2005), corneal epithelial cells (Lyu and Joo, 2005), and hypertrophic chondrocytes (Kerkela *et al.*, 2001). Recent reports have shown that MMP-12 is also expressed by cultured oligodendrocytes (Larsen and Yong, 2004) and by neurons after neonatal hypoxic-ischemic brain injury (Svedin *et al.*, 2009). However, its cellular expression in normal brain tissue and its physiological functions are not well understood. Our results show that during the first few weeks of postnatal development, MMP-12 was highly expressed in cortical pyramidal neurons, cerebellar Purkinje neurons, and occasionally in oligodendrocytes in striatum. MMP-12 expression was increased in Npc1^{-/-}

mice, as measured by microarrays, RT-PCR, and immunofluorescence. Increased expression of MMP-12 was mostly localized in cell bodies of Purkinje neurons and dendrites of cortical neurons. However, our western blot results showed lower levels of pro-MMP-12 protein with relatively high levels of active MMP-12. Although these results may suggest an increased conversion from inactive to active, the discrepancies between mRNA levels and protein levels as well as between western blot and immunohistochemical results are intriguing. One potential explanation could be due to the fact that Purkinje neurons only account for less than 0.1% of cerebellar cells (Hawkes and Gravel, 1991). In addition, MMPs are regulated at four levels: transcription, compartmentalization, zymogen activation, and enzyme inactivation/degradation (Parks *et al.*, 2004; Ethell and Ethell, 2007). Our results could therefore be accounted for by a combined high transcription rate with rapid zymogen activation of MMP-12 in *Npc1*^{-/-} mice.

Whether altered expression of MMP-12 had any functional consequences was first tested in vitro in PC12 cell-derived neuron-like cells by using an MMP-12 inhibitor. We first showed that down-regulation of NPC1 in neuron-like cells resulted in decreased neuritic growth, confirming the important role of NPC1 in neuritic growth; we then showed that MMP-12 inhibition significantly reversed the reduction in axon length elicited by the lack of NPC1. In our subsequent in vivo experiments, we showed that chronic treatment with an MMP-12 inhibitor in *Npc1*^{-/-} mice significantly mitigated axonal pathology. Collectively, our results suggest that increased MMP-12 activity in *Npc1*^{-/-} mice during early postnatal development might contribute to early onset axonal degeneration. However, how *Npc1* deficiency results in MMP-12 overactivation and how overactivation of MMP-12 results in axon degeneration are currently unknown.

Several recent studies have suggested that MMP-12 is involved in oligodendrocyte maturation (Larsen and Yong, 2004; Larsen *et al.*, 2006) and in multiple sclerosis (MS) (Ulrich *et al.*, 2006; Vos *et al.*, 2003). A recent study showed that MMP-12 knockout in mice reduced Theiler's murine encephalomyelitis (TME)-induced demyelination, macrophage infiltration, and motor deficits (Hansmann *et al.*, 2012), further supporting the role of MMP-12 in MS. However, the exact mechanism underlying MMP-12-induced demyelination and axonal degeneration is not clear. In chronic MS, EAE, and cuprizone-induced demyelination animal models, increased MMP-12 expression has been reported mostly in microglia/macrophages, suggesting that MMP-12 may cause demyelination and axonal degeneration by enhancing inflammation (Skuljec *et al.*, 2011; Ulrich *et al.*, 2006; Vos *et al.*, 2003). However, demyelination has also been recently identified in the grey matter in the neocortex of chronic MS patients, suggesting that different mechanisms could account for tissue injury in white and grey matter (Albert *et al.*, 2007; Lassmann and Lucchinetti, 2008; Stadelmann *et al.*, 2008). In particular, cortical pathology in MS has been assumed to be associated with direct damage to neurons and synapses, but with reduced inflammation and gliosis, and increased myelin repair rather than white matter lesions (Albert *et al.*, 2007; Peterson *et al.*, 2001). Interestingly, MMP-12 expression is also up-regulated in grey matter in MS mouse models, suggesting that MMP-12 may be involved in gray matter injury (Skuljec *et al.*, 2011). Similarly, our results revealed increased MMP-12 expression in neurons in cortex and cerebellum in *Npc1*^{-/-} mice. It is thus conceivable that

MMP-12 may be directly involved in axonal injury in *Npc1*^{-/-} mice by disrupting extracellular matrix and damaging axons and oligodendrocytes. It is also possible that MMP-12 secreted by neurons activates microglia, which in turn induces demyelination and axonal degeneration, since we previously showed that microglia-mediated inflammation is present during early postnatal development (Baudry *et al.*, 2003).

Increased MMP-12 expression in Purkinje neurons was associated with increased EMMPRIN, a cell-surface glycoprotein found in normal CNS tissue from embryonic day 13.5 in mice (Fan *et al.*, 1998). EMMPRIN is expressed in many brain structures including hippocampus, cerebellum, cerebral cortex and other regions. EMMPRIN has been proposed to participate in tumor cell survival, invasion, and neuroinflammatory reactions (Muramatsu and Miyauchi, 2003; Agrawal and Yong, 2010). EMMPRIN induces the expression of several MMPs, including MMP-1, -2, -3, -9, and -11 (Biswas *et al.*, 1995; Guo *et al.*, 1997). Notably, increased EMMPRIN expression has been reported in brain samples of MS patients, particularly in plaque-containing areas (Agrawal *et al.*, 2011). Whether EMMPRIN also contributes to MMP-12 up-regulation and whether the two proteins interact in axon degeneration in NPC remain to be elucidated.

Conclusion

The present study revealed a significant increase in the expression of MMP-12 in cortical neurons and cerebellar Purkinje neurons during early postnatal development, before the onset of overt axonal degeneration in *Npc1*^{-/-} mice, suggesting that abnormal MMP-12 activation might participate in the initiation of axonal degeneration. The potential roles of MMP-12 in axonal injury were further supported by cell culture results indicating that MMP-12 inhibition prevented neuritic growth impairment induced by siRNA knockdown of NPC1. Furthermore, our *in vivo* study showed that chronic treatment with an MMP-12 inhibitor significantly reduced axonal pathology in *Npc1*^{-/-} mice. Therefore, our findings provide evidence that MMP-12 plays an important role in NPC pathogenesis, and that inhibition of MMP-12 may be a potential approach for preventing axonal damage and demyelination in NPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MMP-12 is highly expressed in developing cortical neurons and cerebellar Purkinje neurons.
- MMP-12 mRNA levels are increased in cerebellum of *Npc1*^{-/-} mice.
- MMP-12 protein levels are increased in cortical and Purkinje neurons in *Npc1*^{-/-} mice.
- MMP-12 inhibition ameliorates NPC1-deficiency-induced axonal pathology.

Figure 1. MMP-12 protein levels in cerebellum of Npc1^{-/-} and wild-type mice

Representative images of western blots (A) and quantitative results (B) for total and active isoforms of MMP-12, as well as ratios of active over total MMP-12 are shown. While the total level of MMP-12 was decreased (* $p < 0.05$, $n=3$), the ratio of active over total levels was significantly increased in mutant mice, as compared to wild-type (* $p < 0.05$, $n=3$). GAPDH was used as a loading control.

Figure 2. MMP-12 expression in cortex and cerebellum of *Npc1*^{-/-} and wild-type mice
 Coronal forebrain and sagittal cerebellar sections from 15- or 30-day old *Npc1*^{-/-} and wild-type (WT) mice were processed for immunofluorescent staining, as described in Material and Methods, and examined using a confocal microscope. Shown are representative images of MMP-12 immunostaining in cortex (A) and cerebellum (B). Scale bar=20 μ m in A and 10 μ m in B.

Figure 3. MMP-12 expression in striatum of Npc1^{-/-} and wild-type mice

A. Representative images of MMP-12 and SMI-312 (axon marker) immunostaining in 7 day-old Npc1^{-/-} and wild-type (WT) mice. Note that MMP-12 immunoreactivity is mainly found in SMI-312-labeled axon bundles in caudate putamen. Scale bar=100 μ m. **B.** Representative images of MMP-12 and SMI-312 immunostaining in 15 and 30 day-old Npc1^{-/-} and WT mice. **C.** Quantitative analysis of SMI-312-labeled areas in caudate putamen. (n=3–6, *p<0.05 compared to WT mice).

Figure 4. EMMPRIN expression in cerebellum of *Npc1*^{-/-} and wild-type mice

EMMPRIN expression in cerebellum was determined by immunofluorescent staining. **A.** Representative images of EMMPRIN immunostaining in Purkinje neurons of *Npc1*^{-/-} and wild-type (WT) mice. EMMPRIN immunoreactivity was increased in Purkinje neurons of *Npc1*^{-/-} mice. Scale bar=10 μ m. **B.** Co-localization of EMMPRIN with MMP-12 was evident in Purkinje neurons at P15. Scale bar=10 μ m.

Figure 5. Effects of Npc1 siRNA knockdown on neuritic growth and rescue effects of MMP-12 inhibition in PC12 neurons

PC12 cells, cultured and differentiated with nerve growth factor, as described in Materials and Methods, were transfected with GFP combined with either Npc1 siRNA or scrambled control siRNA (Cont). Four hours after transfection, vehicle or MMP-12 inhibitor (MI, 300 ng/ml) was added; cells were analyzed 9 days later. **A.** Representative images of differentiated neuron-like cells from different treatments. Scale bar = 100 μm. **B.** Quantitative analysis of neuritic length from different experimental groups. siRNA knockdown of Npc1 reduced neuritic length, while inhibition of MMP-12 rescued neuritic extension; shown are means $\pm$ SEM from four sets of individual experiments. ** $p < 0.01$, as compared with control siRNA; # $p < 0.05$, as compared with siRNA treatment alone.

Figure 6. MMP-12 inhibition reduced axonal pathology in striatum in developing Npc1^{-/-} mice Immunostaining was performed with anti-neurofilament (SMI-312) antibodies in coronal brain sections from WT or Npc1^{-/-} mice treated with vehicle or MMP-12 inhibitor (MI). **A.** Representative images containing axon bundles in the caudate putamen. Scale bar = 100 μ m. **B.** Quantification of SMI-312-immunoreactive (SMI-312-ir) areas (average size of bundles) in images shown in A. ** indicates $p < 0.01$, as compared to WT mice and ## indicates $p < 0.01$, as compared to vehicle treated Npc1^{-/-} mice (n=7 WT vehicle; n=3 WT MI; n=4 Npc1^{-/-} vehicle; n=5 Npc1^{-/-} MI).

Table 1
Microarray analysis of MMP expression in cerebellum of *Npc1*^{−/−} and wild-type mice

Total RNA extraction and microarray analyses using two platforms were performed as previously described (Liao *et al.*, 2010). **A.** Fold changes of gene expression detected with the Agilent microarray platform in the cerebellum of *Npc1*^{−/−} mice as compared to that in wild-type mice. (n=4 for each group). **B.** Fold changes in gene expression detected with the Illumina microarray platform (n=4 for each group).

A. Agilent			
GENENAME	DESCRIPTION	FC	P value
Mmp12	matrix metalloproteinase 12	5.781	0.001
Mmp3	matrix metalloproteinase 3	2.525	0.150
Mmp7	matrix metalloproteinase 7	1.251	0.639
Mmp2	matrix metalloproteinase 2	1.086	0.618
Mmp9	matrix metalloproteinase 9	−1.339	0.167
Mmp21	matrix metalloproteinase 21	−1.467	0.233
Mmp16	matrix metalloproteinase 16	−2.036	0.403

B. Illumina			
GENENAME	DESCRIPTION	FC	P value
Mmp12	matrix metalloproteinase 12	4.115	0.000
Mmp3	matrix metalloproteinase 3	1.078	0.373
Mmp7	matrix metalloproteinase 7	1.110	0.661
Mmp2	matrix metalloproteinase 2	1.168	0.209
Mmp9	matrix metalloproteinase 9	−1.294	0.014
Mmp16	matrix metalloproteinase 16	−1.529	0.162
Mmp1a	matrix metalloproteinase 1a (interstitial collagenase)	−1.576	0.104