

# Peripheral neuropathy in the Twitcher mouse involves the activation of axonal caspase 3

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## ABSTRACT

Infantile Krabbe disease results in the accumulation of lipid-raft-associated galactosylsphingosine (psychosine), demyelination, neurodegeneration and premature death. Recently, axonopathy has been depicted as a contributing factor in the progression of neurodegeneration in the Twitcher mouse, a *bona fide* mouse model of Krabbe disease. Analysis of the temporal-expression profile of MBP (myelin basic protein) isoforms showed unexpected increases of the 14, 17 and 18.5 kDa isoforms in the sciatic nerve of 1-week-old Twitcher mice, suggesting an abnormal regulation of the myelination process during early postnatal life in this mutant. Our studies showed an elevated activation of the pro-apoptotic protease caspase 3 in sciatic nerves of 15- and 30-day-old Twitcher mice, in parallel with increasing demyelination. Interestingly, while active caspase 3 was clearly contained in peripheral axons at all ages, we found no evidence of caspase accumulation in the soma of corresponding mutant spinal cord motor neurons. Furthermore, active caspase 3 was found not only in unmyelinated axons, but also in myelinated axons of the mutant sciatic nerve. These results suggest that axonal caspase activation occurs before demyelination and following a dying-back pattern. Finally, we showed that psychosine was sufficient to activate caspase 3 in motor neuronal cells *in vitro* in the absence of myelinating glia. Taken together, these findings indicate that degenerating mechanisms actively and specifically mediate axonal dysfunction in Krabbe disease and support the idea that psychosine is a pathogenic sphingolipid sufficient to cause axonal defects independently of demyelination.

Key words: apoptosis, caspase 3, dying-back pathology, Krabbe disease, leukodystrophies, myelin, Twitcher mouse.

## INTRODUCTION

Krabbe disease or globoid cell leukodystrophy is a rare autosomal recessive disease caused by the deficiency of  $\beta$ -GALC (galactosylceramidase) which results in the accumulation of galactosylsphingosine (psychosine) (Igisu and Suzuki, 1984; Suzuki, 1998; Wenger et al., 2000). Most commonly, Krabbe disease is an infantile variant form where symptoms include muscle rigidity and atrophy, hearing and vision defects, developmental regression and, ultimately, fatality before 2 years of age (Aicardi, 1993). Demyelination is the most common hallmark of Krabbe disease. However, other neuropathological defects, including axonal dysfunction, have been shown in the Twitcher mouse, a natural mouse model of Krabbe disease (Galbiati et al., 2009; Castelvetri et al., 2011). While new studies are beginning to shed light on the mechanisms of neurodegeneration in the Twitcher mouse, the temporal pattern of axonal degeneration and the pathological mechanism mediating axonal dysfunction are largely uncharacterized.

Axonal loss is considered as the main cause for permanent neurological handicap in many myelin disorders (Bjartmar et al., 1999; Dutta and Trapp, 2007). We found that peripheral axons are abnormally phosphorylated in the Twitcher mouse, with average diameters decreased to approx. 3  $\mu$ m (L. Castelvetri and E. R. Bongarzone, unpublished data). Mutant peripheral axons also develop swellings and varicosities (Castelvetri et al., 2011). These structural defects parallel developmental delays in mutant mice to acquire postural reflexes, limb strength and maturation of locomotor memory (Olmstead, 1987). All together, these findings indicate a progressive impairment of axonal function, which may include axonal instability, defective axonal transport, deregulated ion channels function and/or abnormal interaction with myelin sheaths.

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**Abbreviations:** APC, adenomatous polyposis coli; CCT, central conduction time; CNS, central nervous system; CMAP, compound motor action potential; cMEP, cortical motor evoked potential; DAB, diaminobenzidine; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; MCV, motor conduction velocity; NCAM, neural cell adhesion molecule; NF-H, neurofilament heavy chain; PFA, paraformaldehyde; WT, wild-type.

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Caspase 3 is a cysteine effector protease that is activated by upstream proteases during late stages of apoptosis and cell death (Okouchi et al., 2007). Caspase activation may be caused by a variety of stimuli, including mitochondrial dysfunction, oxidative stress and irregular calcium homeostasis (Chan, 2001; Orrenius et al., 2003; Polster and Fiskum, 2004; Culmsee and Landshamer, 2006). Caspase 3 activation has been shown in many neurodegenerative diseases, including: Alzheimer's disease, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced Parkinsonian syndrome and ALS (amyotrophic lateral sclerosis) (Martin, 1999; Turmel et al., 2001; Rohn et al., 2002). Oligodendrocyte precursor cells and neurons treated exogenously with psychosine also showed higher levels of active caspase-3, suggesting that this sphingolipid facilitates apoptosis (Giri et al., 2006; Castelvetri et al., 2011). Interestingly, the protease activity of caspase 3 seems to play a role in axonal dysfunction in trophic deprivation and dendritic pruning (Nikolaev et al., 2009; Schoenmann et al., 2010), axonal degeneration in experimental allergic encephalomyelitis (Ahmed et al., 2002), trauma (Chen et al., 2004), proteolysis of spectrin (Reeves et al., 2010) and retrograde fast axonal transport (Morfini et al., 2007).

In the present study, we examined the temporal activation of caspase 3 in the Twitcher spinal cord and sciatic nerve in the context of demyelination.

## MATERIALS AND METHODS

### Animals

Twitcher heterozygous (C57Bl/6J) and WT (wild-type) (C57Bl/6J) mice (Jackson Laboratory) were maintained under standard housing conditions. The Animal Care and Use Committee at the University of Illinois, Chicago approved all animal experiments. Twitcher mice were identified by PCR as previously described (Dolcetta et al., 2006).

### Antibodies and chemicals

Antibodies used were: total caspase 3 (Cell Signaling), Active caspase 3 (Cell Signaling), Actin (Sigma), MBP (myelin basic protein) (a gift from Dr Anthony Campagnoni, University of California, Los Angeles), APC (adenomatous polyposis coli; Millipore), NF-H (neurofilament heavy chain; Cell Signaling), and neurofilament light chain DA2 (Cell Signaling). Isolectin IB4-Alexa Fluor® 488 was from Invitrogen.

### Electrophysiology

Sciatic nerve MCV (motor conduction velocity) was assessed according to previously described techniques (Dolcetta et al., 2005). Briefly, CMAP (compound motor action potential) was obtained by stimulating the nerve at the ankle and ischiatic

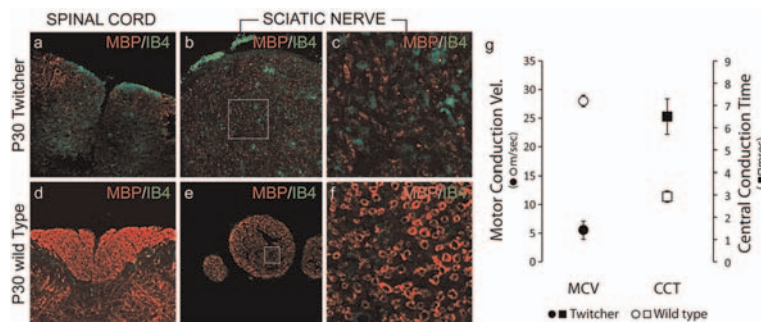
notch with a pair of needle electrodes and recording in distal hind-limb muscles. The active electrode was placed in the middle of plantar muscles, whereas the reference was inserted subcutaneously in the second digit. MCV was measured by dividing the distance between the two points of stimulation by the difference between proximal and distal CMAP latencies. F-waves were recorded from proximal nerve segments and motor roots with the same montage as described for MCV (Toyoshima et al., 1986). cMEPs (cortical motor evoked potentials) were elicited with a pair of needle monopolar electrodes through the mouse primary motor area (Chiba et al., 2003). CCT (central conduction time), an index of the propagation time of stimulus-related volleys descending along corticospinal tracts, was calculated as the difference between the latency of the sMEP (spinal motor evoked potential), i.e. latency of muscle responses to stimulate lumbar motor roots electrically minus that of the cMEP.

### Western blotting

Spinal cord and sciatic nerves were removed from WT and Twitcher mice at postnatal days 7, 14–15 and 30. Tissues were homogenized in protein lysis buffer containing: 20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM PMSF, 2 mM sodium orthovanadate, 1 mM NaF and 300 nM okadaic acid. Samples were sonicated on ice and spun down at 5000 rev./min for 5 min at 4°C. The supernatant was quantified using BCA Bradford assay (Bio-Rad). SDS/PAGE was performed using 4–12% Bis-Tris gels (Invitrogen). Proteins were transferred on to nitrocellulose membranes, blocked in 5% (w/v) non-fat dried skimmed milk powder, 1% BSA, 0.05% Tween 20 in Tris/Glycine buffer and incubated in primary antibodies at 4°C overnight. Membranes were incubated in secondary peroxidase-conjugated antibodies at room temperature for 1 h. Proteins were visualized using enhanced luminescence (Thermo Scientific) and quantified using ImageJ software (NIH).

### Immunohistochemistry

Mice were anaesthetized and perfused with saline followed by 4% (w/v) PFA (paraformaldehyde). Spinal cords and sciatic nerves were extracted and further fixed in 4% PFA for 24 h. Tissue was transferred to 20% sucrose for 24 h then embedded and quickly frozen in OCT (optimal cutting temperature). Cryosections (20 µm) were mounted on super-frost slides and stored at –20°C. Sections were dried at room temperature and washed in PBS. DAB (diaminobenzidine) staining was performed by first quenching sections for 10 min at room temperature using 10% methanol and 10% H<sub>2</sub>O<sub>2</sub> in PBS. ABC secondary antibodies and substrate solution (Vectastain Elite) was employed using manufacturer's guidelines. DAB solution (1%) was used for visualization. Sections were dehydrated using a gradient series of alcohol and xylene and mounted with Permount (Fisher). Sections for immunofluorescence were dried at room temperature, washed in 1 × PBS, and blocked in



**Figure 1** Analysis of Twitcher P30 spinal cord and sciatic nerve. Confocal analysis of MBP and Isolectin-IB4 (IB4) immunostaining in the Twitcher (TWI) (a) and wild-type (WT) (d) spinal cord. Magnification  $\times 100$ . Double labelling in the TWI sciatic nerve (b, c, magnification  $\times 200$ ) reveals MBP loss and microglia activation compared with WT littermate controls (e, f, magnification  $\times 630$ ). Sciatic nerve MCVs and CNS CCTs of the TWI mouse are compared with WT controls (g).

4% BSA, 0.1% Triton X-100/PBS at room temperature for 1 h. Sections were incubated with primary antibodies in blocking solution for 48 h and secondary antibodies (Alexa Fluor<sup>®</sup> 488 and 555) for 2 h. Sections were mounted using Vectashield mounting medium (Vector) and visualized using a Zeiss Meta 510 confocal microscope.

### Cell culture

Motoneuronal NSC34 cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 5% FBS (fetal bovine serum), penicillin/streptomycin (Gibco) and L-glutamine (Gibco). Psychosine (Sigma), C<sub>6</sub>-ceramide (Sigma), and D-sphingosine (Sigma) were resuspended in 0.001% ethanol.

### Immunocytochemistry

Cells were grown on Matrigel<sup>™</sup>-treated coverslips. Cells were fixed in 4% PFA/PBS, permeabilized in 0.1% Triton X-100 and blocked with 4% BSA/PBS. Coverslips were incubated with primary antibodies diluted in 1% BSA/PBS overnight at 4°C. Cells were incubated with secondary antibodies Alexa Fluor<sup>®</sup> 488 and Alexa Fluor<sup>®</sup> 555 in 1% BSA/PBS and mounted.

### DNA fragmentation assay

NSC34 cells were collected by spinning down at 7000 rev./min for 5 min and washed with sterile PBS. DNA lysis buffer (0.5 mg/ml of proteinase K in 1 M Tris, pH 7.9, 2 M NaCl, 500 mM EDTA and 20% SDS) was added to the pellet and incubated at 56°C overnight. After RNase treatment, phenol purification of DNA was performed and 0.8  $\mu$ g of DNA was run on a 2% agarose gel. Ethidium bromide-stained gels were visualized using a Bio-Rad Chemi-Doc XRS with Quantity One 1D gel visualizing software.

### Statistical analysis

Data were analysed using Student's *t* test.  $P < 0.05$  was considered significant. Results are means of independent measurements  $\pm$  S.E.M.

## RESULTS

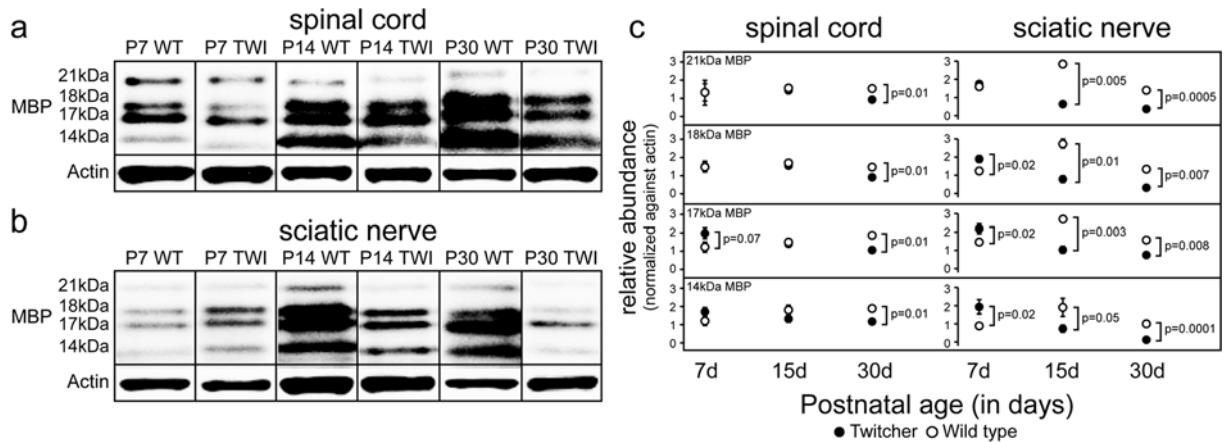
### Temporal analysis of MBP expression in the Twitcher mouse

The Twitcher mouse is a natural occurring demyelinating mouse model of KD where psychosine accumulates in the CNS (central nervous system) and PNS (peripheral nervous system) (Shinoda et al., 1987; Galbiati et al., 2009). Demyelination has previously been shown to begin during the third and fourth weeks in the Twitcher mouse (Nagara et al., 1982). At postnatal day 30, the Twitcher spinal cord has abundant microgliosis and reduction of MBP compared with age-matched controls (Figures 1a and 1d). This is also observed in the Twitcher sciatic nerve at the same age (Figures 1b, 1e, 1c and 1f). Demyelination and the concurrent loss of MBPs and axon insulation become compounding factors that likely lead to slowed nerve conduction seen in the Twitcher mouse. Here, we show a strong decrease in MCVs of the sciatic nerve and an increase in spinal cord conduction latency (Figure 1g). MCVs were  $\sim 30$  m/s in the P30 WT sciatic nerve, compared with  $\sim 5$  m/s in the Twitcher. CCT increased from  $\sim 3$  ms in the wild-type to  $\sim 7$  ms in the Twitcher cord.

To determine the temporal pattern of MBP isoform loss in the Twitcher mouse, spinal cord and sciatic nerves from WT and Twitcher mice at ages P7, P15 and P30 were extracted and homogenized for immunoblot analysis. We show a decrease in all four isoforms (21, 18, 17 and 14 kDa) of MBP in the P30 Twitcher spinal cord (Figures 2a and 2c) but not at P7 and P15. Peripherally, there is a significant decrease in all four MBP isoforms starting at P15 in the sciatic nerve of the Twitcher mouse and continuing to P30 (Figures 2b and 2c). Interestingly, the 14, 17 and 18 kDa MBP isoforms were found significantly increased in the Twitcher mouse compared with WT at 7 days of age (Figure 2C).

### Active caspase 3 in the Twitcher spinal cord

Activation of caspase 3 has been shown to increase in oligodendrocytes exposed to psychosine and in the brain of



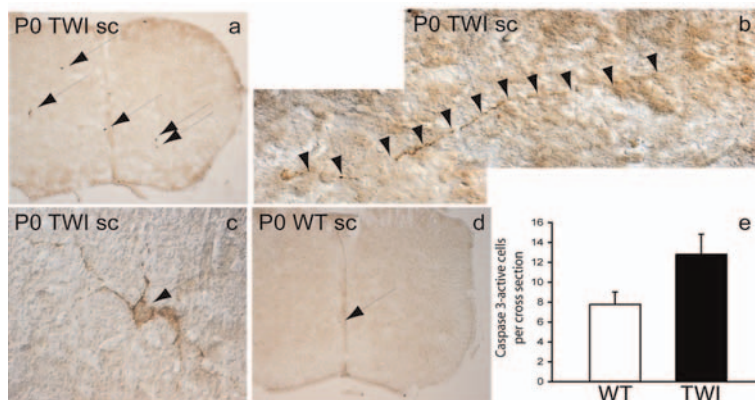
**Figure 2** MBP in the TWI and WT nervous system  
 Immunoblotting analysis of MBP isoforms (21, 18, 17 and 14 kDa) at P7, 14 and 30 of the TWI spinal cord (a) and sciatic nerve (b) compared with WT controls. Quantifications of results show significant loss of all MBP isoforms at later stages in the disease, while MBP isoforms 18, 17 and 14 are increased at P7 (c).

sick Twitcher mice (Giri et al., 2006; Galbiati et al., 2007b). However, caspase 3 activation has not been reported in the spinal cord and nerves of the Twitcher mouse. Caspase 3 activation is a natural process to eliminate unnecessary cells during embryonic development. Interestingly, increased numbers of neurons containing active caspase 3 were found in the Twitcher spinal cord as early at birth (Figures 3a–3c and 3e). Importantly, while active caspase 3 was observed in cell bodies, it was also found in cell processes and axons in the Twitcher spinal cord (Figure 3c and arrowheads in Figure 3b).

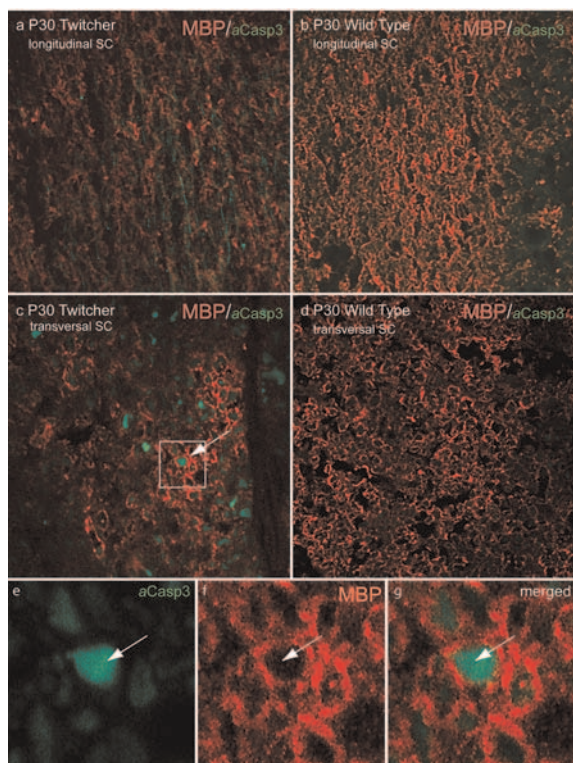
Next, we examined the level of active caspase 3 in the spinal cord at later stages of the disease. Figure 4 shows positive staining of caspase 3 in longitudinal sections of the

Twitcher spinal cord compared with controls (Figures 4a and 4b). Interestingly, the strongest staining was localized to the ventral funiculus region, which contains the anterior corticospinal and lateral vestibulospinal tracts, better seen in cross-sections in Figures 4(c) and 4(d). Active caspase 3 was observed clearly localized in axons (arrows in Figures 4e and 4g). Finally, the relative abundance of active caspase 3 in the cord was determined by quantitative immunoblotting (Figure 5a). This analysis confirmed that active caspase 3 is significantly increased in the Twitcher mouse spinal cord at P30 (Figure 5b) but not at earlier time points.

Finally, we examined what cell types had active caspase 3 levels in the spinal cord. Immunohistochemical analyses showed that active caspase 3 was localized primarily to neuronal



**Figure 3** Immunohistochemistry of TWI spinal cord at P0  
 Active caspase 3 is visible in the TWI spinal cord (a). Caspase 3 is visible in neuronal processes (b) and cell bodies (c). This is compared with normal caspase 3 activity observed in the P0 WT mice (d). Quantifications show elevated caspase 3 levels at birth (e). Magnifications (a, d)  $\times 50$ ; (b, c)  $\times 400$ .



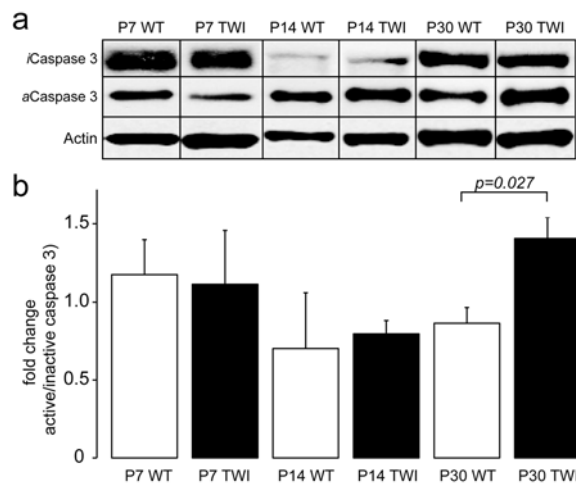
**Figure 4 Active caspase 3 in the Twitcher spinal cord**

Active caspase 3 ( $\alpha$ Casp3) (green) in longitudinal sections of the P30 Twitcher spinal cord (a) compared with WT (b). Magnification  $\times 200$ . Active caspase 3 in transverse sections of the ventral funiculus region of the spinal cord shows caspase 3 in the Twitcher (c) and WT (d). Magnification  $\times 400$ . Active caspase 3 is localized to some axons ensheathed with MBP (red). Magnification  $\times 1000$ .

processes at P30 (Supplementary Figure S1 available at <http://www.asnneuro.org/an/003/an003e066add.htm>). Co-localization with NF-H further showed that caspase 3 was affecting neuronal axons (Supplementary Figure S1, arrows). We did not find significant levels of active caspase 3 staining in oligodendrocytes, which were identified by reaction with the antibody against APC, a marker for mature oligodendrocytes (Supplementary Figure S2, white arrows; available at <http://www.asnneuro.org/an/003/an003e066add.htm>). Active caspase 3 was observed to co-localize sporadically with GFAP (glial fibrillary acidic protein)-positive astroglial cells (Supplementary Figure S2, blue arrow).

### Active caspase 3 in the Twitcher sciatic nerve

To determine whether apoptotic pathways are activated in the PNS of the Twitcher, mouse sciatic nerves were also analysed for active caspase 3 levels. Confocal microscopy showed positive caspase 3 staining at all three time points in the Twitcher nerves; however, reactivity became stronger only in P15 and P30 nerves of the Twitcher mice (Figures 6a and 6c, compared with littermate controls in Figures 6b and 6d). Interestingly, active



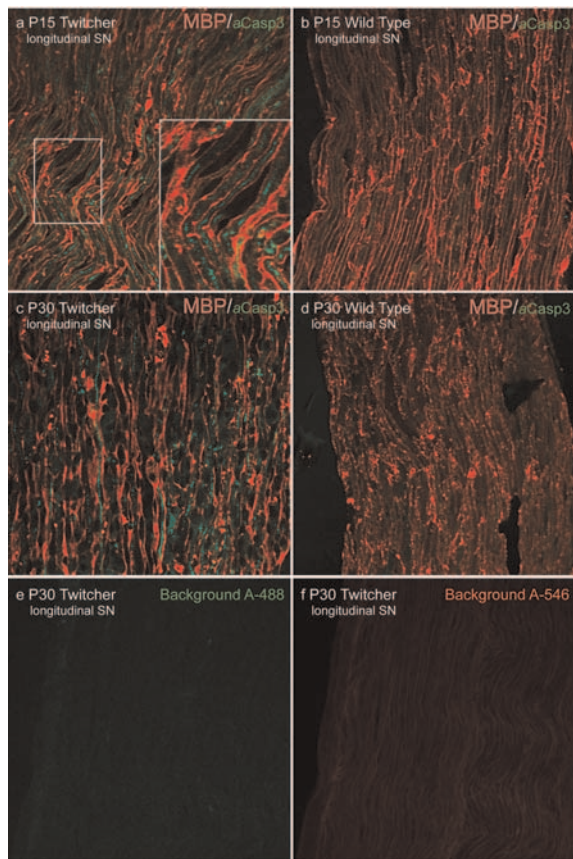
**Figure 5 Immunoblot analysis of active caspase 3 in the spinal cord**

Inactive (iCaspase 3) and active caspase 3 ( $\alpha$ Caspase 3) levels are shown in WT and TWI spinal cord tissue at P7, 14 and 30 (a). Quantifications of fold change in active/inactive caspase 3 levels are shown (b).  $n=3$ .

caspase 3 was strongly localized to axons (myelin sheaths were marked by immunostaining against MBP, in red; Figure 6a). Immunoblot analysis of nerve extracts (Figure 7a) confirmed a significant increase in active caspase 3 levels in the sciatic nerve starting at P14 and continuing throughout the disease (Figure 7b). Interestingly, active caspase 3 was significantly lower in mutant nerves at P7 (Figure 7b). Altogether, these results suggest that, while the time points of significant increase in active caspase 3 levels correlate with the loss of myelin integrity in the sciatic nerve, activation of caspase 3 in axons may also occur independently of demyelination.

### Psychosine is sufficient to activate caspase 3 in cultures of a motor neuronal cell line in the absence of myelinating glia

Finally, to test if psychosine is a pathogenic molecule sufficient to activate caspase 3 in neurons, we cultured differentiated motor neuronal NSC34 cells in the presence or absence of psychosine and in the absence of myelinating glial cells. Incubation with 1  $\mu$ M psychosine (Whitfield et al., 2001) for 24 h led to significant activation of caspase 3 (Figures 8a and 8b). Increasing psychosine levels to 5  $\mu$ M further elevated caspase levels, showing a dose-dependent response. This level of caspase activation was similar that obtained when NSC34 cells were incubated with 30  $\mu$ M  $C_6$ -ceramide, a strong activator of caspases (Spinedi et al., 1998; Ravid et al., 2003). Immunocytochemical detection of active caspase 3 in NSC34 cells (in green, Figure 8c) was observed in the cytoplasm and neuronal processes of NSC34 cells (identified with antibodies against neurofilament light chain, in red) 2 h after treatment with 5  $\mu$ M psychosine (Figure 8c). Exposing NSC34 cells to psychosine for 2 h led to DNA fragmentation (Figure 8e).



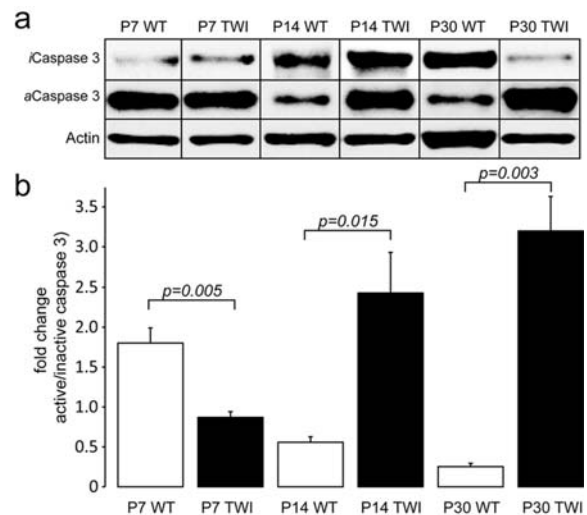
**Figure 6 Active caspase 3 in the TWI sciatic nerve**  
Caspase 3 (green) is localized to nerve fibres with different stages of MBP loss (red) in the P15 TWI mouse (a, inset) compared with P15 WT mice (b). This continues in the P30 TWI (c) compared with controls (d). Background levels for TWI (A-488) (e) and TWI (A-546) (f) animals are also shown. Magnification  $\times 200$ .

## DISCUSSION

Structural defects in axons of the Twitcher mouse suggesting a dying-back neuropathy were previously observed by our group (Castelvetri et al., 2011). We report here for the first time that activation of pro-apoptotic protease caspase 3 is highly increased in central and peripheral mutant axons before any sign of activation of the protease in neurons or even glial bodies, providing further support to the hypothesis of a dying-back mechanism. Our study also found unprecedented early increases of some MBP isoforms, suggesting abnormal myelination in peripheral nerves early after birth.

### Early increases in MBP expression suggest abnormal myelination in the young sciatic nerve

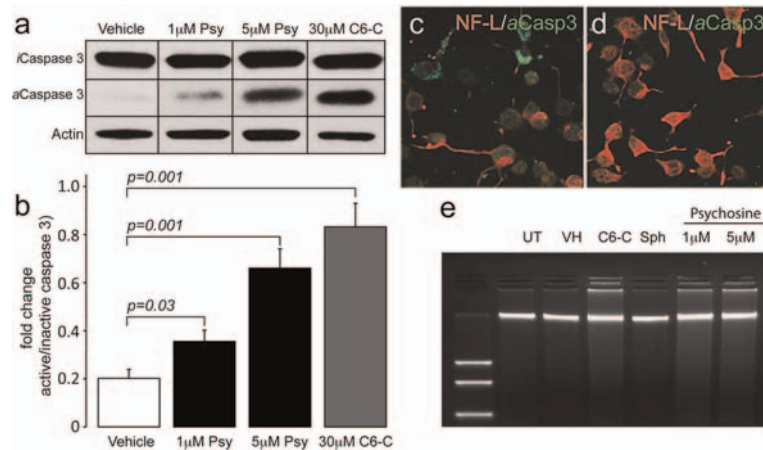
Ever since Knud H. Krabbe described the first five cases of this lysosomal storage disease in 1916 (Krabbe, 1916) much work has been done to determine the underlying



**Figure 7 Immunoblot analysis of active caspase 3 in the sciatic nerve**  
Inactive (iCaspase 3) and active caspase 3 ( $\alpha$ Caspase 3) levels are shown in WT and TWI sciatic nerve tissue at P7, 14 and 30 (a). Quantifications of fold change in active/inactive caspase 3 levels are shown (b).  $n=3$ .

pathogenic mechanism of this fatal disease. A central role in pathogenesis has been attributed to psychosine (Suzuki, 1998), a galactosylsphingolipid that rapidly accumulates in lipid rafts (White et al., 2009) and interferes in multiple signalling pathways (Giri et al., 2006, 2008; White et al., 2011). The Twitcher mouse, a natural mutant murine model of Krabbe disease, has been an invaluable resource to understand these processes, including demyelination and other gliotic responses (Kobayashi et al., 1980; Takahashi and Suzuki, 1984; Mikoshiba et al., 1985). From these pioneering studies, it was concluded that myelination in the Twitcher mouse is normal for the first 2–3 weeks of life, and demyelination strikes later, between P20 and P25 (spinal cord) and  $\sim$ P20 (peripheral nerves).

In order to understand the correlation between demyelination, axonal damage and neuronal death, we first studied the effects of disease on the abundance of each MBP isoform. Products of the MBP gene are fundamental components of peripheral and central myelin (Barbarese et al., 1978; de Ferra et al., 1985; Boggs, 2006; Harauz et al., 2009). MBP loss accompanies myelin degeneration in demyelinating diseases such as multiple sclerosis and metachromatic leukodystrophy diseases (Gendelman et al., 1985; Ramakrishnan et al., 2007). Loss of function of the MBP gene in the Shiverer mouse also causes a myelin formation defect (Bird et al., 1978). Because of the functional impact of MBPs on myelin function and their specificity to mark myelin sheaths, the abundance of MBPs has been a very useful biochemical endpoint to study myelin loss (Takahashi and Suzuki, 1984). Albeit the loss of MBPs as a general marker of myelin has been evaluated in sick Twitcher mice (Mikoshiba et al., 1985), individual measurement of MBP isoforms has not been reported for the Twitcher. Our studies showed significant decreases for each



**Figure 8** **Caspase 3 in vitro**  
Immunoblot analysis of inactive and active caspase 3 levels in NSC34 cells treated with 1 and 5 μM psychosine (PSY) where 30 μM C<sub>6</sub>-ceramide (C6-C) serves as the positive control (a). Quantifications of fold change in active/inactive caspase 3 levels are shown (b) ( $n=2$ ). Active caspase 3 (aCasp3, green) is visible in 5 μM 24 PSY treated cells for 2 h (c) compared with vehicle controls also labelled with neurofilament light chain (NF-L, red). Magnification  $\times 200$ . (d). DNA fragmentation assay shows an increase in DNA fragments when cells are treated with 1 and 5 μM PSY compared with untreated (UT), vehicle (VH) and 1 μM d-sphingosine (Sph) negative controls where C6-C serves as a positive control (e).

MBP isoform in the lower spinal cord only in 30-day-old mutant but not in younger animals. Instead, reductions of all MBP isoforms in the sciatic nerve were significant at P15, earlier than previously described (Takahashi and Suzuki, 1984). These data confirm that demyelination in the Twitcher mutant begins as a peripheral insult  $\sim 10$  days before central damage. Unexpectedly, the abundance of the 14, 17 and 18.5 kDa isoforms of MBP was significantly increased in mutant sciatic nerves at P7. In the cord, the 14 and 17 kDa MBP isoforms were slightly increased as well, but in a non-significant manner. This finding suggests either an increase in Schwann cell proliferation or in the synthesis of peripheral myelin. Previous studies using *in vivo* [<sup>3</sup>H]thymidine metabolic labelling showed a dysfunction in Twitcher Schwann cells, which continued to proliferate in mutant nerves even after demyelination (Komiya and Suzuki, 1994). The reason for this defect has never been clarified. We have previously shown that psychosine accumulates in lipid rafts of the mutant brain and sciatic nerve, disrupting raft architecture (White et al., 2009). Accumulation of psychosine in P7 mutant sciatic nerves is low in comparison with late stages of the disease but significantly higher than age-matched WT nerves (L. Castelvetri and E. R. Bongarzone, unpublished data). One possible interpretation is that psychosine may exhibit dual effects in a concentration-dependent manner: at low concentrations, psychosine may facilitate myelination and/or Schwann cells proliferation, likely facilitating raft-mediated trophic signalling such as the PDGF (platelet-derived growth factor) pathway, relevant for Schwann cell proliferation (Yamada et al., 1996). As disease progresses and psychosine accumulates, it becomes a neurotoxic sphingolipid, activating death pathways in mature Schwann cells and causing demyelination.

### Caspase 3 is activated in mutant axons independently of neuronal cell death

Various studies have shown evidence of an ongoing axonal and neuronal dysfunction during demyelination in Krabbe disease (Hogan et al., 1969; Jacobs et al., 1982; Dolcetta et al., 2006; Galbiati et al., 2007a, 2007b, 2009; Escolar et al., 2009; Hofling et al., 2009; Castelvetri et al., 2011). Interestingly, axonal counting performed on cross-sections of Twitcher sciatic nerves, perhaps the best region in this mutant where to study axonal and myelin damage, showed the absence of axonal 'loss' (Jacobs et al., 1982). However, a recent re-evaluation of this issue using longitudinal sections of mutant nerves clearly showed the presence of structural alterations, including swellings and breaks at early postnatal stages (Castelvetri et al., 2011). This last study also showed absence of significant levels of neuronal cell death in the spinal cord of the mutant, at least until very late in the disease. In all, one possible interpretation is that a dying back neuropathy affects this mutant, with caspase 3 activation and damage to some axons occurring before neuronal death.

To examine this possibility in more detail, immunoblotting analyses for measuring changes in caspase activation were performed and showed elevated levels of active caspase 3 in mutant sciatic nerves as early as P15. This was coincident with the sharp decrease in MCV (Dolcetta et al., 2006) the infiltration of macrophages (Ohno et al., 1993; Taniike and Suzuki, 1994) and demyelination. Furthermore, our analyses demonstrated that in P15 mutant sciatic nerves active caspase 3 was mostly localized within axons. Active caspase 3 was marginal or absent in neuronal bodies in the cord or Schwann cell bodies in the nerve at P15. In fact, apoptotic cell bodies were observed only at later stages using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end

labelling) and therefore would not co-localize with active caspase 3 at these ages (Castelvetri et al., 2011). Some staining of caspase 3 was localized to GFAP positive astrocytes. This likely represents sporadic apoptotic astroglial death. Its contribution to the phenotype observed in the Twitcher mouse is unknown. While the expectation was to find oligodendroglia also expressing active caspase 3, our results showed very little if any of this caspase active in this cell type. At late stages of disease, oligodendrocytes may die of apoptosis (Bjartmar et al., 1999; Taniike et al., 1999; Castelvetri et al., 2011). These results may indicate that the death of oligodendrocytes in the Twitcher mouse may be a late event in the disease, involving other apoptotic/degenerative pathways. Active caspase 3 seemed to accumulate in myelinated and demyelinated axons (axons with strong punctate MBP immunostaining pattern), suggesting that myelin is not a primary trigger for activation of the caspase pathway in axons. Unmyelinated sciatic nerve C-fibres (Kobayashi et al., 1988; Tanaka et al., 1989) may be undergoing dying-back death in the Twitcher mouse along with fibres that have undergone demyelination or even before demyelination begins. Intriguingly, not all axons showed active caspase 3, which also indicates that activation of the protease is selective to a subset of fibres. An area of growing interest in axonal degeneration is the role that microgliosis plays in regulating axonal-myelin interactions. Our studies show uneven distribution of macrophages (detected by binding of IB4 lectin) throughout the nervous system. The reason for this distribution is not fully understood, although it may be caused by localized cross-talk between macrophages and the damaged tissue. Macrophages may well exert differential distress/trophic signalling cascades, executing death pathways in some cell types (Burguillos et al., 2011) while aiding in debris removal and repair in others (Kondo et al., 2011). Further studies need to address whether infiltrating macrophages in the Twitcher nerve regulate the activation of caspase 3 in axons.

In the spinal cord, active caspase 3 staining was localized in axons of the ventral funiculus region of the spinal cord, the area containing the anterior corticospinal tract and the lateral vestibulospinal tract. These tracts are descending motor pathways responsible for maintaining motor control of the lower limbs (Ludolph et al., 1987; Ugawa et al., 1991). The presence of active caspase 3 in these axons suggests axonal distress, which may be partly responsible for the defects seen in the hind limbs in the Twitcher mouse. The molecular mechanism by which caspase may exert a pathogenic effect in the Twitcher axon is the current object of studies in our laboratory. Caspase 3-mediated axonopathy may involve axonal transport deregulation, proteolysis of cytoskeletal components such as spectrin and microtubule-associated Tau and/or deregulation of downstream proteases (Gamblin et al., 2003; Reeves et al., 2010; Westphal et al., 2010). Immunostaining for caspase in the cord of newborn mutant detected also increased levels of caspase 3 positive axons in the neonatal cord, indicating a premature level of axonal stress in neonates. The relevance of this finding is unclear, but it may 'prime' or 'mark' axons for further structural damage seen at later stages. As psychosine builds up

and accumulates to toxic levels, neurons appear to activate caspase 3, which eventually trigger an apoptotic-death programme.

In conclusion, the work presented here shows activation of axonal death in the form of active caspase 3 in the Twitcher PNS (peripheral nervous system) and CNS. Our study demonstrates that psychosine is sufficient to cause activation of this protease in the absence of myelinating glia, strongly underlining the potential role of this toxin in triggering this pathogenic mechanism. The link between psychosine and the defect described in this study could also involve other partners such as ER (endoplasmic reticulum) stress, imbalance of calcium or abnormal levels of ion channels (Orrenius et al., 2003; Kurnellas et al., 2005). Mitochondrial dysfunction and sequential cytochrome c release also can increase caspase 3 activation (Polster and Fiskum, 2004). Taken together, our observations suggest a form of 'dying back' neuropathy in the nervous system of the Twitcher mouse, likely regulated by the levels of toxic psychosine, in parallel with abnormal myelination during early stages of the disease.

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