

The Sphingolipid Psychosine Inhibits Fast Axonal Transport in Krabbe Disease by Activation of GSK3 β and Deregulation of Molecular Motors

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Loss of function of galactosylceramidase lysosomal activity causes demyelination and vulnerability of various neuronal populations in Krabbe disease. Psychosine, a lipid-raft-associated sphingolipid that accumulates in this disease, is thought to trigger these abnormalities. Myelin-free *in vitro* analyses showed that psychosine inhibited fast axonal transport through the activation of axonal PP1 and GSK3 β in the axon. Abnormal levels of activated GSK3 β and abnormally phosphorylated kinesin light chains were found in nerve samples from a mouse model of Krabbe disease. Administration of GSK3 β inhibitors significantly ameliorated transport defects *in vitro* and *in vivo* in peripheral axons of the mutant mouse. This study identifies psychosine as a pathogenic sphingolipid able to block fast axonal transport and is the first to provide a molecular mechanism underlying dying-back degeneration in this genetic leukodystrophy.

Introduction

Krabbe disease is an autosomal-recessive leukodystrophy caused by the loss of function of galactosylceramidase (GALC), a lysosomal enzyme that catabolizes galactosylated sphingolipids. Psychosine (galactosyl-sphingosine) is a lipid-raft-associated neurotoxin (White et al., 2009) known to accumulate and to trigger pathogenesis in Krabbe patients (Igisu and Suzuki, 1984; Suzuki, 1998). The diffuse damage to myelin sheaths, a hallmark of the disease, is thought to result from dysfunction of both oligodendrocytes and Schwann cells consequent to the accumulation of psychosine (Tanaka et al., 1989; Jatana et al., 2002). Krabbe patients also show progressive and severe neurological deficiencies in both the central and peripheral nervous systems. Symptoms include muscle rigidity and atrophy, ataxic movement, hearing and vision defects, and rapid loss of cognitive and motor skills (Duffner et al., 2009; Escobar et al., 2009). The pathogenic mechanisms mediating these deficiencies are unknown, but the disease presents as a dying-back degeneration of mutant axons (Castelvetri et al., 2011) and involves dysfunction of selective neuronal populations (Krabbe, 1916).

Axonal dystrophy in conjunction with increased phosphatase (PP1 and PP2A) and caspase-3 activity lead to defects in cytoskeletal components in the twitcher mouse, a well characterized animal model of Krabbe disease (Castelvetri et al., 2011; Smith et al., 2011; Cantuti-Castelvetri et al., 2012). The selectivity (i.e., the disparity in which neuronal and axonal populations are affected), the dying-back pattern affecting axons, and the characteristic appearance of axonal swellings point to defects in fast axonal transport (FAT). The physiological consequences of deficient FAT have been established for several neurological disorders (Pigino et al., 2003; Morfini et al., 2004, 2006, 2009a,b; Ori-McKenney et al., 2010). However, the pathogenic role of defective FAT in a leukodystrophy such as Krabbe disease had not been addressed previously.

This study demonstrates that psychosine inhibits FAT and requires the activation of axonal phosphotransferases PP1 and GSK3 β and the abnormal phosphorylation of the molecular motor protein kinesin light chains (KLCs). *In vitro* and *in vivo* neuroprotection experiments demonstrated the pathogenic relevance of this pathway in neurodegeneration in Krabbe disease.

Materials and Methods

Animals. Breeder twitcher heterozygous mice (C57BL/6J, twi/+) were originally purchased from The Jackson Laboratory and maintained under standard housing conditions. Protocols were according to animal care and use committee protocols of our institution. The twitcher mutation was analyzed as described previously (Sakai et al., 1996). Male and female twitcher mice were used indistinctly.

Cell cultures. Cortical neurons were prepared as described previously (Kaech and Banker, 2006). Briefly, cortices were isolated from E16 C57BL embryos. Tissue was minced, treated with 0.25% trypsin, and passed through a fire-polished pipette. Cells were then plated in DMEM

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(Mediatech) supplemented with 10% FBS for 2 h and further incubated in neurobasal medium supplemented with B27. NSC34 cells were grown in 5% FBS DMEM, L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen). NSC34 cells were serum deprived for 12 h before treatments. Glass coverslips were precleaned with nitric acid, water, and ethanol and then autoclaved before use. Lipids were purchased from Sigma. Enantiomeric psychosine was obtained from M.S. and resuspended in ethanol. Psychosine was added to *in vitro* cell systems at 1–5 μM , which falls within the range of concentrations measured in the central and peripheral nervous systems of the twitcher mouse (1–10 nmol/g tissue or 1–10 μM ; Kobayashi et al., 1987; White et al., 2009). Okadaic acid (Calbiochem) was added together with psychosine, whereas TDZD8 or L803 inhibitors (Calbiochem) (Leopold et al., 1992; Chen et al., 2004; Watzlawik et al., 2010) were added 30 min before the addition of psychosine. Psychosine treatment lasted for 3 h. The final concentration of ethanol was maintained at 0.01% v/v.

Mitochondrial motility measurements. Cells were treated as described above and incubated at 37°C for 20 min with 75 nM Mitotracker G diluted in growth medium. After replenishing with fresh medium, live video recording (1 frame every 10 s for 10 min) was performed using an AxioCam Hrm camera attached to an Axiovert 200M microscope (both Carl Zeiss). To measure the speed and number of the moving/stationary mitochondria, kymographs were generated and analyzed using ImageJ. The vehicle of psychosine (0.01% ethanol in DMEM) was used as control. Each condition was tested three times on separate dates.

Transmission electron microscopy. Tissue was fixed in 2% paraformaldehyde, 0.5% glutaraldehyde overnight at 4°C. Samples were postfixed for 1 h in 2% OsO_4 in 0.1% sodium cacodylate, dehydrated in graded steps of acetone, and embedded in Araldite. Ultrathin sections (60 nm) were cut with a DiATOME diamond knife on a Leica Ultracut UCT microtome, collected on Formvar-coated one-hole grids, and counterstained with uranyl/lead. Samples were observed in a JEOL 1220 electron microscope.

Western blotting. Tissues were homogenized in lysis buffer (1 mM PMSF, 2 mM sodium orthovanadate, 1 mM NaF, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl_2 , 300 mM okadaic acid), briefly sonicated on ice, and centrifuged at 5000 rpm for 5 min to remove debris. Protein content was quantified using the Bradford assay (Bio-Rad). Equal amounts of proteins (10–20 μg) were loaded on a 4–12% Bis-Tris gel (Invitrogen) and electrophoresed at 80 mV for 4 h before transfer of gels on a PVDF membrane (Bio-Rad) for 2 h. Membranes were blocked in 5% nonfat milk, 1% BSA, 0.05% Tween 20 in Tris-glycine buffer (blocking solution) and probed with primary antibodies at 4°C overnight. After incubation with secondary HRP-conjugated antibodies, bands were visualized using the enhanced luminescence kit (Thermo Scientific) and quantified using ImageJ. Antibodies used detected the following: actin (rabbit; Sigma), heat shock protein 60 (HSP60; mouse; Santa Cruz Biotechnology), SNAP 25 (goat; Abcam), phosphorylated ser-9 GSK3 β (rabbit; Cell Signaling Technology), total GSK3 β (rabbit; Cell Signaling Technology), kinesin heavy chain (KHC; mouse; H2), KLC (mouse; L2), and dephosphorylated KLC (mouse; 63-90). The latter three antibodies were donated by S.T.B. For the quantification of the 63-90 antibody (dephosphorylated KLC), bands were normalized to KHC using ImageJ. Although total KLC is a more appropriate loading control, normalization toward total KHC or KLC gave the same results. For the quantification of total protein levels, actin was used as a loading control.

Vesicular axonal transport in sciatic nerve. To analyze for the accumulation of transported cargoes, spinal cord and distal portions of sciatic nerves and proximal stumps from ligated sciatic nerves (Hirokawa et al., 1991) were processed for immunoblotting.

Vesicle motility assays. Axoplasm was extruded from giant axons of the squid *Loligo pealei* (Marine Biological Laboratory) as described previously (Brady et al., 1982). Sphingolipids were diluted into X/2 buffer (175 mM potassium aspartate, 65 mM taurine, 35 mM betaine, 25 mM glycine, 10 mM HEPES, 6.5 mM MgCl_2 , 5 mM EGTA, 1.5 mM CaCl_2 and 0.5 mM glucose, pH 7.2) supplemented with 2–5 mM ATP and 20 μl of this mixture was added to perfusion chambers. Preparations were analyzed on a Zeiss Axiom microscope with a 100 \times , 1.3 numerical aperture ob-

jective, and DIC optics. Hamamatsu Argus 20 and model 2400 CCD cameras were used for image processing and analysis. Organelle velocities were measured with a Photonics Microscopy C2117 video manipulator (Hamamatsu) by matching calibrated cursor movements to the speed of vesicles moving in the axoplasm. Individual organelles are below the resolution limit and their individual properties (frequency of pauses, number of moving organelles) cannot be analyzed. However, the average velocity measurements were correlated with the number of vesicles moving in a given treatment: low rates reflect reduced numbers of vesicles and slower mean velocities (Brady et al., 1993; Ratner et al., 1998). This method reliably reproduces the rates of transport of individual organelles moving on axoplasms.

In vivo administration of GSK3 β inhibitor. After genotyping, twitcher pups were injected intraperitoneally with a myristoylated form of L803 (a GSK3 β inhibitor) or vehicle (3 mice/group) beginning on postnatal day 3 and daily thereafter until postnatal day 20. Each injection delivered 400 nM (Wang et al., 2008) of the inhibitor in a 25 μl bolus of vehicle (saline). Motor conduction velocities were recorded in sciatic nerves before tissue collection as described previously (Dolcetta et al., 2005) and axonal transport analysis was done as described above.

Statistical analysis. Results are expressed as mean \pm SE in three to six independent experiments. Data were analyzed by two-tailed unpaired *t* test (confidence interval 95%) or by ANOVA test followed by the Newman–Keuls multiple-comparison test to analyze for all possible comparisons, where applicable. *p* < 0.05 was considered statistically significant.

Results

Psychosine inhibits FAT through PP1 and GSK3 β

Recent descriptions of axonal degeneration in twitcher axons suggest that selected populations of neurons undergo a dying-back degeneration (Castelvetti et al., 2011; Smith et al., 2011; Cantuti-Castelvetti et al., 2012). Axonal swellings are pathognomonic of structural and physiological deficiencies in diseased axons, which may include focal accumulation of unsorted cargoes due to defects in FAT (Coleman and Ribchester, 2004; De Vos et al., 2008). We investigated whether psychosine is a pathogenic compound able to interfere with FAT using *ex vivo* axoplasm preparations from *L. pealei*, a model that has been used successfully for quantitative real-time analyses of the effects of bioactive molecules on both anterograde and retrograde components of FAT (Brady et al., 1982). Extruded axoplasms were perfused with 1 or 5 μM psychosine and anterograde and retrograde rates were recorded during 50 min. Psychosine induced a significant (*p* = 0.001) inhibitory response in both FAT components (Fig. 1*a,b*), whereas axoplasms perfused with D-sphingosine, the sphingoid base of psychosine (Fig. 1*c*), or with an enantiomeric form of psychosine (Fig. 1*d*) had no effect on FAT.

Various signaling pathways have been reported to regulate FAT (Morfini et al., 2002; 2006; Pignino et al., 2009). A signaling pathway involving the activities of PP1 and GSK3 β regulates FAT by phosphorylation of KLCs (subunits of the motor kinesin) (Morfini et al., 2004) and promotes release from its transported cargoes (Morfini et al., 2002). To determine whether this pathway was involved in psychosine-mediated inhibition of FAT, motility assays were performed by coprefusing psychosine with okadaic acid and inhibitor 2, which are selective inhibitors of PP1. Interestingly, both inhibitors significantly (*p* = 0.001) protected anterograde and retrograde components of FAT (Fig. 1*e,f,i,j*). Similarly, ING-35, a specific inhibitor of GSK3 β , almost completely abolished (*p* = 0.001) the effect of psychosine (Fig. 1*g,i,j*). To evaluate the role of alternative pathways, inhibitors of other kinases, such as the casein kinase 2 inhibitor tetrabromocinnamic acid (Martin and Pagano, 1994), were coprefused with psychosine, but these inhibitors did not exhibit a significant protective effect (Fig. 1*h*). These experiments indicate that psycho-

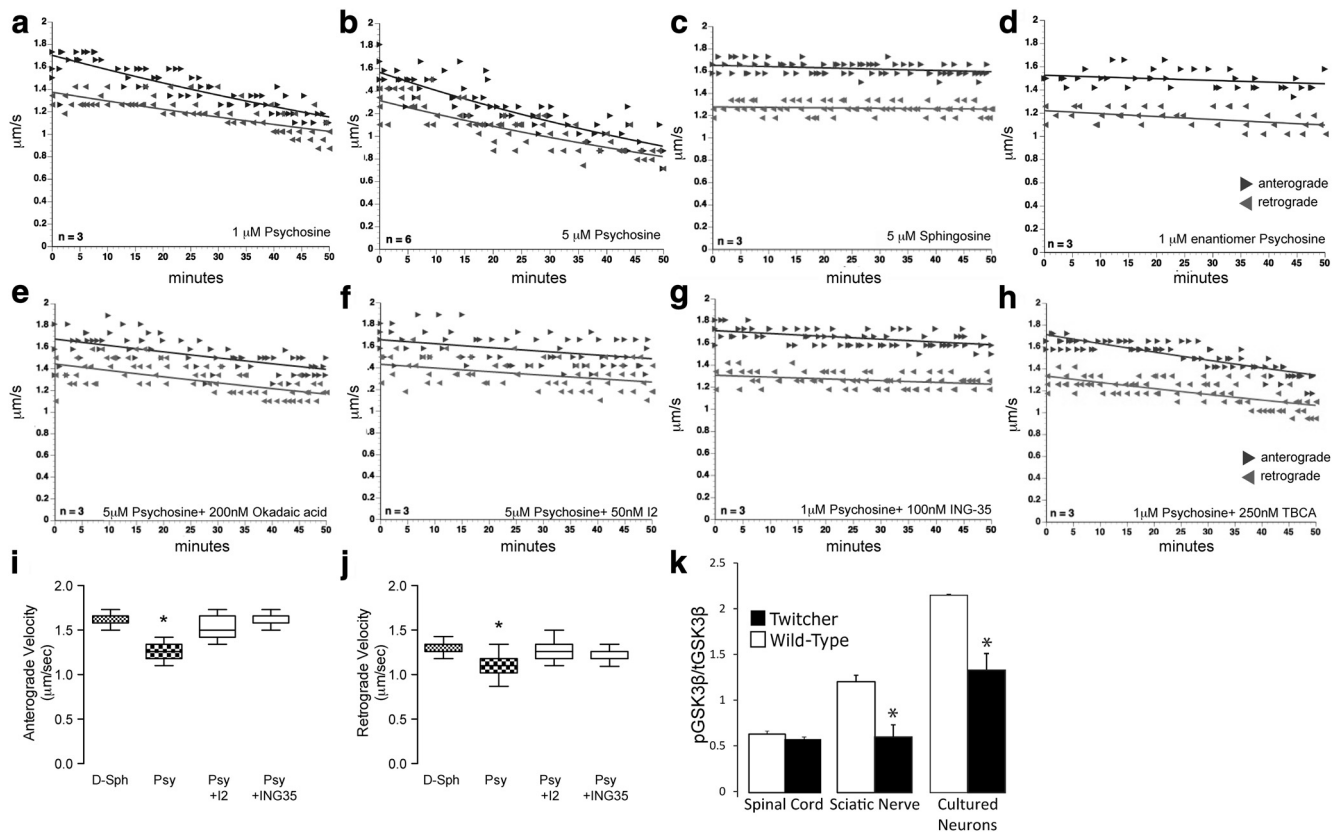


Figure 1. Psychosine is a pathogenic lipid that inhibits fast axonal transport through PP1 and GSK3 β activities. Measurements of FAT rate ($\mu\text{m/s}$) for membrane-bound organelles were performed on squid axoplasms perfused with psychosine or control lipids. Black arrowheads and line and gray arrowheads and line represent anterograde and retrograde FAT components, respectively. Perfusion of 1 μM (**a**) and 5 μM (**b**) psychosine into squid axoplasms led to a strikingly reduced rate of anterograde and retrograde transport. The effects of 1 and 5 μM psychosine were comparable, suggesting that much lower concentrations may also affect FAT. Perfusion with 5 μM D-sphingosine (**c**) or 1 μM enantiomeric psychosine (**d**) had no effect on transport ($n = 3$ –6 axoplasms). Coperfusion of 5 μM psychosine with 200 nM okadaic acid (a general phosphatase inhibitor) (**e**) or 50 nM inhibitor 2 (I2) (a selective PP1 inhibitor) (**f**) led to protection of transport rates. Coperfusion of 1 μM psychosine with 100 nM ING-35 (a selective GSK3 β inhibitor) (**g**) led to an almost complete protection of FAT, whereas coperfusion of 1 μM psychosine with 250 nM tetrabromocinnamic acid, a selective casein kinase II inhibitor (**h**), did not protect against the effects of psychosine. Anterograde (**i**) and retrograde (**j**) transport rates at the end of the treatments indicated a significant recovery ($p = 0.001$, ANOVA) in axoplasms coperfused with the inhibitors I2 and ING 35. Immunoblotting with antibodies against phosphorylated (ser-9) GSK3 β (pGSK3 β) and total GSK3 β (tGSK3 β) revealed significant differences ($p = 0.04$, unpaired t test) in activated (dephosphorylated) GSK3 β levels in 30-day sciatic nerves and 10-day cultured primary cortical neurons, but not in extracts from 30-day spinal cords ($n = 6$ per condition per group) (**k**).

sine impairs FAT mainly through deregulation of PP1 and GSK3 β activities.

Nerve tissue from twitcher mutants contain higher levels of activated GSK3 β

Activation of GSK3 β is regulated primarily by phosphorylation-dephosphorylation of ser-9, a process mediated by a variety of kinases including Akt (Woodgett, 1994) and phosphatases including PP1 (Morfini et al., 2002). We previously demonstrated the presence of increased PP1 activity levels in twitcher mutants (Cantuti-Castelvetti et al., 2012), which is consistent with the prediction of higher levels of GSK3 β activity *in vivo*. Immunoblot analyses for phosphorylated (ser-9) and total GSK3 β revealed a decrease in the level of the phosphorylated (inactive) kinase in peripheral (sciatic) nerves, but not in the spinal cord of twitcher mice (Fig. 1*k*). Differences in GSK3 β activity in these two regions of the nervous system are likely due to the ubiquitous nature of this kinase in the nervous system, including both neuronal and non-neuronal cells. This ubiquity may mask changes in neuronal activity that occurred in the spinal cord. To analyze GSK3 β phosphorylation levels in neurons, we acutely isolated neurons from twitcher cortices. Indeed, mutant neurons contained significantly lower levels of phosphorylated (ser-9)

GSK3 β than wild-type neurons (Fig. 1*k*), which is consistent with our observations in nerve tissue and *ex vivo* axoplasm analyses. These data suggest that the deficiency of GALC and the subsequent accumulation of psychosine are correlated with higher levels of activated GSK3 β .

KLC is less abundant and more phosphorylated in mutant axons

Activation of the axonal PP1-GSK3 β pathway by psychosine predicts an increase in phosphorylated KLC and the premature release of their cargoes. To test this hypothesis, we first used immunoblotting to assess levels of KHCs and KLCs in postmortem nerve tissues isolated from affected mutants. We found no significant differences in the abundance of either kinesin in twitcher spinal cord extracts at 7 or 30 d of age (Fig. 2*a,c,e*), whereas both kinesins were significantly decreased ($p = 0.03$) in 30-day twitcher sciatic nerve extracts (Fig. 2*b,d,f*). This strong decrease in kinesins suggests a reduced trafficking of motors in twitcher mice during the onset of neurological manifestations (~postnatal day 25), which is consistent with a distal axonopathy.

To determine whether phosphorylation of KLCs was changed *in vivo*, we examined tissue extracts by immunoblot-

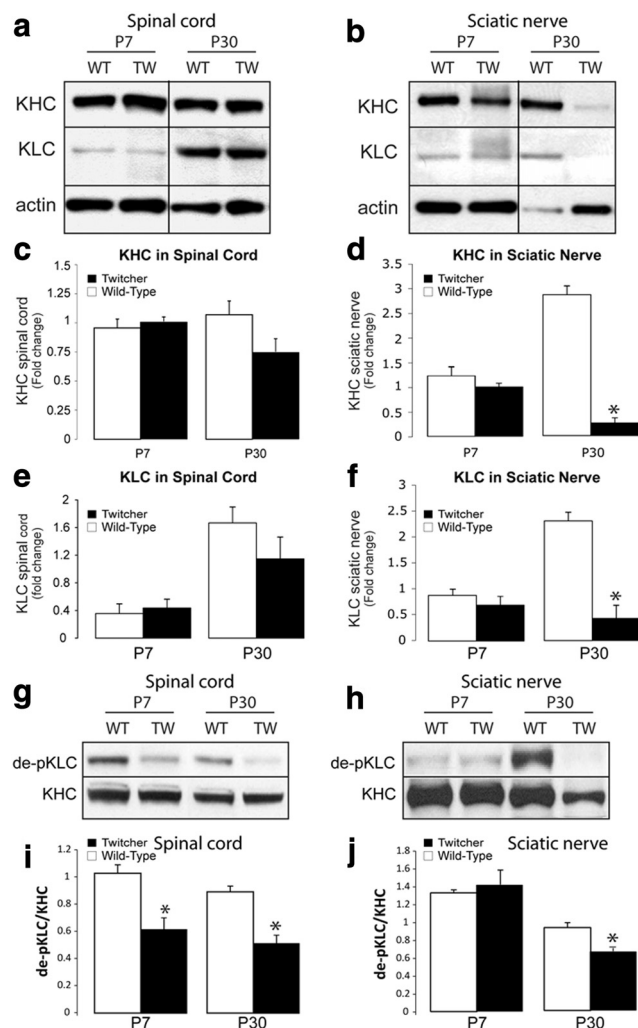


Figure 2. Alteration in molecular motor kinesins in twitcher sciatic nerves. Immunoblotting analyses for total KHC and KLC to test for changes in relative amounts of these motors 7- and 30-day (P7 and P30) spinal cords (**a**) and sciatic nerves (**b**) ($n = 4$ /condition/group; actin used as loading control) revealed no significant differences in either KHC (**c**) and KLC (**e**) in spinal cord extracts from the mutants at either time point, although the P30 time point shows a trend of reduced amount of these motors ($p > 0.05$; t test unpaired). Levels of KHC (**d**) and KLC (**f**) differed significantly ($p = 0.03$, unpaired t test) in P30 twitcher sciatic nerves but were less affected in P7 mutant sciatic nerves. Immunoblotting analyses using monoclonal antibody 63-90, which detects changes in phosphorylation of KLC by GSK3 β in 7- and 30-day-old (P7 and P30) spinal cords (**g**) and sciatic nerves (**h**) ($n = 4$ /condition/group), revealed significant ($p = 0.002$ – 0.015 , unpaired t test) differences in the level of dephosphorylated KLC (de-pKLC) in all twitcher samples except in P7 mutant sciatic nerves (**i,j**).

ting with the 63-90 antibody (Stenoien and Brady, 1997), which recognizes changes of phosphorylation in this motor protein (Morfini et al., 2004). Analyses showed a strong decrease in the immunoreactivity of monoclonal antibody 63-90 (binding of the antibody is inversely proportional to phosphorylation) in spinal cord (Fig. 2*g,i*; $p = 0.002$) and sciatic nerves (Fig. 2*h,j*; $p = 0.01$), particularly from 30-day-old twitcher mice. This finding is consistent with the increase in activated GSK3 β (Fig. 1) and PP1 activity (Cantuti-Castelvetri et al., 2012). Significant ($p = 0.015$) phosphorylation of KLCs was also observed in cord extracts from 7-day-old twitcher mice, indicating an early deregulation of motors in this mutant.

In vivo and *in vitro* FAT is reduced in twitcher neurons

To determine whether twitcher mutants develop axonal transport defects *in vivo*, we measured transport efficiency by biochemical analysis of accumulated cargoes in wild-type and twitcher mice after sciatic nerve ligation. This simple paradigm has been used successfully for many years to measure changes in transport (Hirokawa et al., 1991), providing one of the first demonstrations that FAT existed. Proximal (relative to the spinal cord) stumps of the nerve (~ 1 mm) were used for immunoblotting analyses. Measurement of the relative accumulation of molecular motor KHC, mitochondrial molecular chaperone HSP60 (Walls et al., 2012), and t-SNARE protein SNAP-25 (Walch-Solimena et al., 1995) indicated a significant ($p = 0.001$) decrease in their abundance in 7- and 30-day-old mutant proximal stumps (Fig. 3*a,b*). Transmission electron microscopy showed clear accumulations of vesicular bodies in all of the wild-type control nerves analyzed (Fig. 3*g*, inset), whereas mutant nerves showed a mix of axons without any visible accumulation of vesicular bodies (Fig. 3*d*) and with accumulated material (Fig. 3*e*). Vesicular structures were observed on the cytoplasmic face of mutant axoplasm (Fig. 3*c*, arrows), which were absent in wild-type axoplasm (Fig. 3*f*). The presence of these vesicular accumulations suggests alterations in the sorting of transported membranous organelles (Edgar et al., 2004), which is consistent with impaired FAT.

Two experiments were performed to determine whether deficits in FAT are due to a cell-autonomous mechanism. First, wild-type cortical neuronal cultures were exposed to psychosine and the anterograde and retrograde movement of fluorescently labeled mitochondria was measured. Kymographs ($n = 30$ per group) determined that psychosine reduced both the number (Fig. 4*a,b*) and speed (Fig. 4*c,d*) of anterograde and retrograde moving mitochondria. Second, similar mitochondrial mobility analyses were performed on cultures of mutant neurons. Kymograph analyses showed that twitcher neurons displayed comparable deficits in anterograde and retrograde moving mitochondria (Fig. 4*e,f*) and reduced speeds (Fig. 4*g,h*), indicating that mutant neurons are autonomously affected.

Neuroprotection of axonal transport by interference of GSK3 β activity

We previously reported that PP1 activity is elevated in nerve tissues of the twitcher mouse and that this is a contributing factor to cytoskeletal abnormalities and the dying-back pathology detected in this Krabbe disease model (Cantuti-Castelvetri et al., 2012). Further, psychosine was sufficient to stimulate higher activities of PP1 in the motor-neuron-like cell line NSC34 (Cantuti-Castelvetri et al., 2012) and to inhibit FAT through PP1 and GSK3 β (Fig. 1). Our *ex vivo* axoplasm experiments using PP1 and GSK3 β inhibitors provided further evidence that this effect is specific, suggesting that FAT might be protected from psychosine effects with appropriate neuroprotective compounds. To test this hypothesis further and to evaluate potential clinical applications, we conducted *in vitro* and *in vivo* neuroprotective experiments using inhibitors of PP1 and GSK3 β .

First, activation of PP1 was induced on NSC34 cells as described previously (Cantuti-Castelvetri et al., 2012) and the level of downstream activation of GSK3 β through PP1 was assessed by immunoblotting in the presence and absence of okadaic acid. Okadaic acid was sufficient to abolish psychosine-triggered activation of GSK3 β (Fig. 5*a,b*), indicating the need for PP1 in activating GSK3 β dephosphorylation. To examine the functional relevance of interfering with PP1 and GSK3 β after psychosine

treatment, we tested for changes in the antibody 63-90 immunoreactivity of KLC in extracts of treated NSC34 cells. As expected, psychosine alone induced a significant decrease in the immunoreactivity of kinesin to antibody 63-90 (Fig. 5*a*). Co-treatment of neurons either with okadaic acid to inhibit PP1 or with TDZD8 to inhibit GSK3 β completely abolished the effect of psychosine on kinesin phosphorylation (Fig. 5*a,c*).

To test the functional impact of interfering with GSK3 β on mitochondrial motility, NSC34 cells were coincubated with psychosine and a cell-permeable inhibitor of GSK3 β myristoylated L803. Kymograph analyses of fluorescently labeled mitochondria showed that L803 was sufficient to restore mitochondrial transport in psychosine-treated cells (Fig. 5*d–g*). Control experiments with cells treated only with L803 showed no significant change in mitochondrial motility.

To evaluate the *in vivo* effects of interfering with GSK3 β , twitcher mice were injected intraperitoneally with myristoylated L803 in saline from day 2 of age until day 20. Mutant mice were not conditioned with any other treatment nor did they receive traditional bone marrow transplantation to correct for the biochemical defect in psychosine catabolism. Extracts from nerves were used to measure for changes in 63-90 immunoreactivity. Untreated mutants showed the expected decrease in 63-90 immunoreactivity (Fig. 6*a*), whereas mutant mice treated with the inhibitor revealed significant increases in 63-90 immunoreactivity (Fig. 6*a*; $p = 0.01$).

To test for possible protection against axonal transport defects in twitcher mice treated with the GSK3 β inhibitor, we measured the percent recovery of the transport of kinesin and HSP60 in ligated sciatic nerves. Administration of the L803 inhibitor led to a significant ($p = 0.01$) recovery in the amount of transported kinesin and HSP60 (Fig. 6*b*). Finally, we examined the extent to which L803 treatment ameliorated some of the clinical symptoms of mutant mice. Because this treatment was not combined with any kind of metabolic correction (e.g., bone marrow transplantation or gene therapy; Galbiati et al., 2009), L803-treated twitcher had no recovery in psychosine or GALC levels (data not shown) and their life span was not expected to be affected. Measurement of motor conduction velocity in sciatic nerves showed a mild but not significant improvement in treated mutants (Fig. 6*c*). However, these results are encouraging because they show that this axonal defect may be treated positively *in vivo* using pharmacological strategies and underline the potential benefit of combinatorial treatments. Further experiments will determine the synergy of these neuroprotective therapies in combination with bone marrow transplantation and gene therapy.

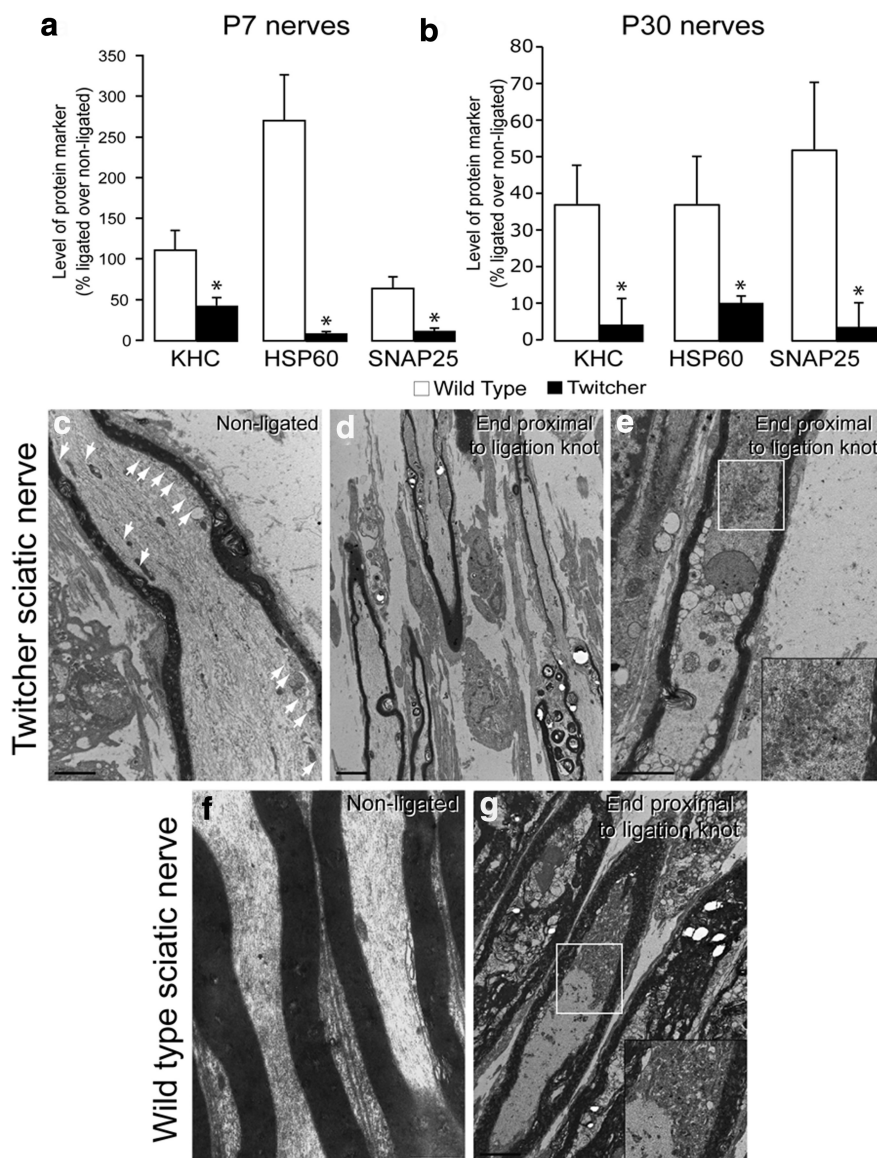


Figure 3. Defective axonal transport in the twitcher sciatic nerve. Immunoblotting analyses of the proximal end of ligated nerves in 7-day (P7) (*a*) and 30-day (P30) (*b*) twitcher mice ($n = 6$ independent ligations) for KHC, HSP60 (a mitochondrial marker), and SNAP 25 revealed significantly reduced ($p = 0.003$ – 0.048 , unpaired t test) accumulation of each marker at both time points. Transmission electron microscopic images of nonligated and ligated twitcher (*c–e*) and wild-type (*f,g*) nerves (ligation site on the top right corner) revealed abundant accumulation of vesicular material toward the site of ligation in wild-type axons (inset in *g*), whereas markedly fewer axons with accumulated material (*d,e*, inset in *e*) were seen in the ligated mutant nerve. Bar indicates 5 μ m. Arrows in *c* point to vesicular material underneath the mutant axolemma.

Discussion

Since its description in 1916 (Krabbe, 1916), neuronal vulnerability has been an unclear aspect of Krabbe disease, but recent work is shedding light on the underlying pathogenic mechanism(s) (Castelvetri et al., 2011; Smith et al., 2011; Cantuti-Castelvetri et al., 2012). The present study provides further evidence that Krabbe disease develops defects in vesicular transport and identifies psychosine as the first pathogenic sphingolipid inhibiting FAT through deregulated activities of PP1-GSK3 β .

FAT ensures neuronal communication, regulating the timely delivery of cargoes to neuronal domains. The pathogenic relevance of impaired transport is best exemplified by the discovery of specific mutations in molecular motors in hereditary spastic

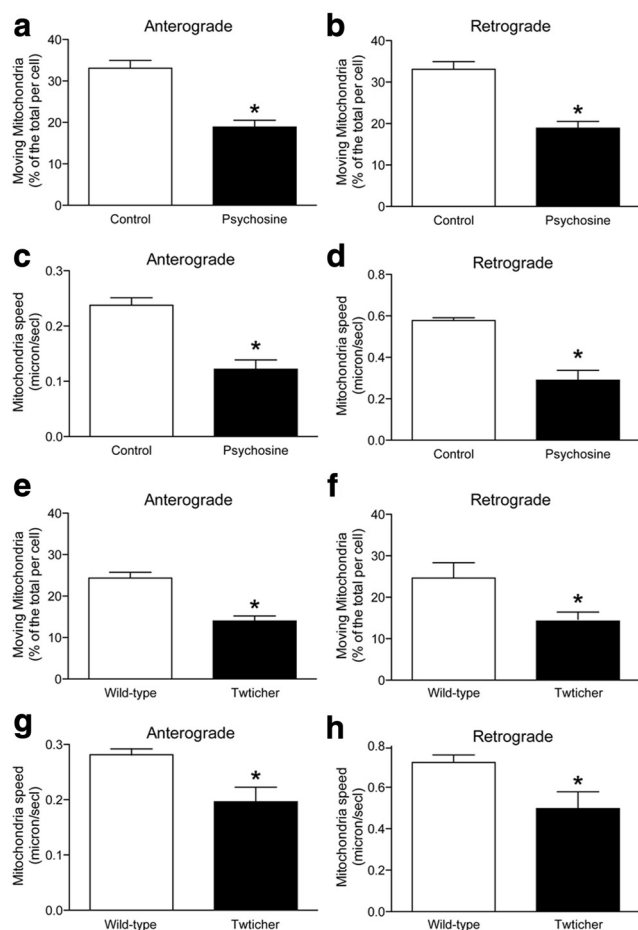


Figure 4. Defective transport of mitochondria in psychosine-elevated conditions. Anterograde and retrograde movement of fluorescently labeled mitochondria was measured using kymograph analyses. Wild-type neurons exposed to 1 μ M psychosine are shown to have significantly ($p = 0.01$, unpaired t test) fewer mitochondria moving either anterogradely (**a**) or retrogradely (**b**) compared with the control (vehicle treatment, 0.01% ethanol in DMEM). Kymographs permitted the measurement of significant ($p = 0.02$, unpaired t test) reductions in anterograde (**c**) and retrograde (**d**) average moving speeds. Significant ($p = 0.01$, unpaired t test) reductions in moving mitochondria (**e,f**) and movement speed (**g,h**) were measured in cultures containing twitcher neurons.

paraplegia HSP10 and some forms of sensory and motor neuron disease (Zhao et al., 2001; Reid et al., 2002; Puls et al., 2003). These deficiencies decrease cargo transport, initiating an axonal dying-back pathology that kills vulnerable neurons. Therefore, dysfunctional FAT might be seen as a common pathogenic mechanism activated in several unrelated neurological diseases. In the case of Krabbe disease, a deficit of FAT likely adds to the severity of demyelination, leading to a complex neurological phenotype. It is possible to speculate how deficient kinesin-based delivery affects neurons. For example, impaired mitochondrial transport could lead to inefficient energy distribution along the axon, reducing its capacity to maintain physiological ion concentrations. Higher influx of calcium through the sodium-calcium exchanger (Craner et al., 2004) and subsequent activation of calcium-dependent proteases may contribute to the axonal swellings (Beirrowski et al., 2005; Castelvetti et al., 2011). Similarly, defective transport of ion channels will affect the functionality of nodes of Ranvier, impairing nerve conduction. Kinesin-based transport is also fundamental for the delivery of myelin components (Carson et al., 1997; Lyons et al., 2009). Therefore, defects in motor-based-transport such as those described in this study may also

occur in other cell types such as oligodendrocytes and Schwann cells.

Because myelination is a known regulator of axonal transport (de Waegh and Brady, 1990; de Waegh et al., 1992), our results do not exclude the possibility that abnormal myelination might contribute to FAT defects. However, demyelination effects on FAT are likely to be secondary to direct effects of psychosine, given that twitchers show normal myelination during early postnatal life (Nagara et al., 1982) and that our *in vitro* studies using twitcher neurons confirmed a cell autonomous defect. However, after disease onset and with ongoing demyelination, FAT defects are likely exacerbated by demyelination. Psychosine inhibits specifically both anterograde and retrograde components of FAT. The GSK3 β pathway has been primarily associated with regulation of anterograde transport (Morfini et al., 2004). However, our studies using GSK3 β inhibitors revealed an additional regulatory role on retrograde transport. Further studies are needed to characterize the molecular partners mediating this effect.

In vivo, transport defects were correlated with the progressive accumulation of psychosine (Igisu and Suzuki, 1984; Castelvetti et al., 2011). It seems highly unlikely that psychosine inhibits FAT by eliciting nonspecific effects such as detergent action (i.e., disruption of organelle membranes) or by disrupting microtubules because: (1) membranous organelles and microtubules in extruded axoplasms exposed to psychosine were structurally unaffected for the duration of our experiments (data not shown); (2) electron microscopy showed normal structure of membranous organelles and microtubules within twitcher axons; and (3) coprefusion of psychosine with PP1 and GSK3 β inhibitors protected transport significantly. Furthermore, inhibition of FAT by psychosine does not require either myelin or myelinating glia, because motility axoplasm assays and *in vitro* experiments on NSC34 cells and primary cortical neurons were conducted in the absence of mutant myelin and glia. The finding of psychosine in acutely isolated neurons further suggests an *in situ* source of this neurotoxin (Castelvetti et al., 2011).

If psychosine is sufficient to block FAT, it must somehow be spatially related to the transport machinery to exert this inhibitory role. Psychosine may accumulate in the axon from different sources, including neuronal synthesis and transport via membrane-bound cargoes (M.B. Santos and E.R.B., manuscript in preparation), and lipid transfer from myelin sheaths/surrounding glia (Vance et al., 1994, 2000; Krijnse-Locker et al., 1995). To date, it remains unclear whether one or more of these sources contribute to psychosine accumulation in axons *in vivo*.

Krabbe disease is not associated with mutations in molecular motors or cytoskeletal proteins, so FAT inhibition must be caused by one or more pathogenic components intrinsic of the disease. Psychosine, which activates pathogenic responses in glia (Khan et al., 2005; Giri et al., 2006), also increases axonal PP1 and PP2A activities (Cantuti-Castelvetti et al., 2012). Here we found that psychosine exacerbates axonal GSK3 β activity through PP1, a process shown to increase phosphorylation of KLCs, releasing kinesin from cargoes (Morfini et al., 2002). One possible mechanism by which psychosine elicits these changes is through membrane alterations. Because psychosine is a lipid-raft-associated sphingolipid (White et al., 2009; 2011) and because axonal PP1 and GSK3 β colocalize in transported vesicles (Morfini et al., 2004) and associate with rafts (Sui et al., 2006; Henkhaus et al., 2008), psychosine may modify PP1 and/or GSK3 β activities by regulating their interaction with membranes. For example, caveolin-1 is a raft-specific protein that can inhibit PP1 (Hen-

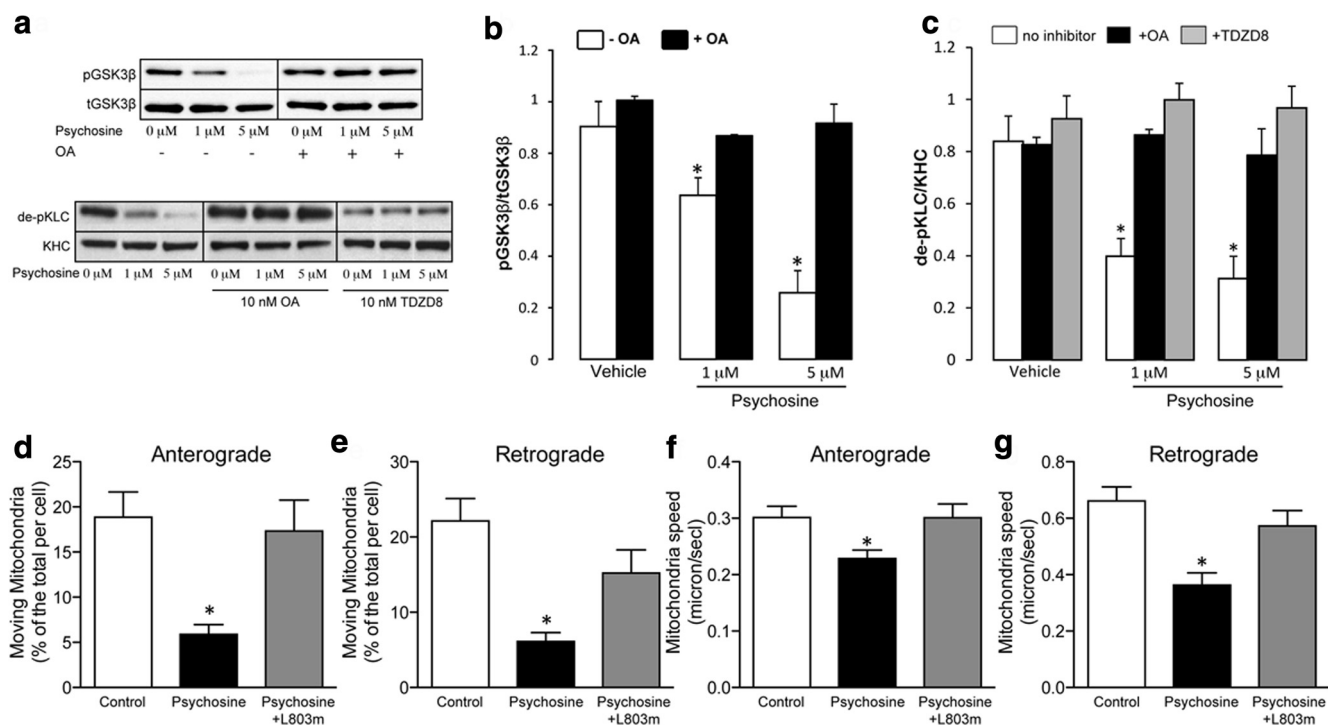


Figure 5. *In vitro* neuroprotection using PP1 and GSK3 β inhibitors. Immunoblotting of NSC34 cells for phosphorylated (ser-9) GSK3 β (pGSK3 β) and for the 63–90 epitope (de-phosphorylated KLC [de-pKLC]) after treatment of cells with 10 nM okadaic acid (a general inhibitor of phosphatases) and TDZD8 (a selective inhibitor of GSK3 β ; **a**) indicated a potent normalization effect by okadaic acid on levels of activated GSK3 β ($p = 0.05$ for 1 μ M psychosine and $p = 0.001$ for 5 μ M psychosine, unpaired t test; **b**). Pretreatment of NSC34 cells with okadaic acid or TDZD8 led to a marked reduction of phosphorylation of KLC (measured as an increase of dephosphorylated KLC) by inhibition of PP1 and GSK3 β (**a,c**), whereas pretreatment of NSC34 cells with myristoylated L803 (a selective GSK3 β inhibitor) significantly ($p = 0.01$, unpaired t test) improved the proportion of mitochondria moving either anterogradely (**d**) or retrogradely (**e**) as well as their anterograde (**f**) and retrograde (**g**) movement speeds. Data are shown as mean \pm SE of three to five independent experiments.

khaus et al., 2008). Psychosine may affect PP1-caveolin-1 in rafts by releasing PP1. Because the PP1/GSK3 β pathway was also found to deregulate FAT in other pathological conditions (Kanaan et al., 2011), these findings suggest that the PP1/GSK3 β pathway is a common mechanism in unrelated neurological conditions.

Although abnormal GSK3 β activity may affect transport of many membrane-bound organelles through deregulation of kinesin-based motors, other organelles such as mitochondria may be targeted through other still unclear mechanisms. For example, recent studies suggest that mitochondria may not require KLCs (Cai and Sheng, 2009) for transport but rather a complex of various docking proteins (Stowers et al., 2002; Guo et al., 2005; Glater et al., 2006), whereas other studies show that both KLC and KHC and GSK3 β are associated with mitochondria (Leopold et al., 1992; Chen et al., 2008). In our experiments, an inhibitor of GSK3 β acted as a neuroprotective agent, improving axonal mobilization of mitochondria and confirming a fundamental role of this pathway in regulating mitochondrial transport. Furthermore, PP1/GSK3 β inhibitors also ameliorated psychosine-inhibitory effects on the retrograde component of FAT, suggesting that the PP1/GSK3 β and/or some other functionally interacting pathways may have regulatory roles in both transport components. These pathways could decrease FAT by modifying other components of the transport machinery, such as

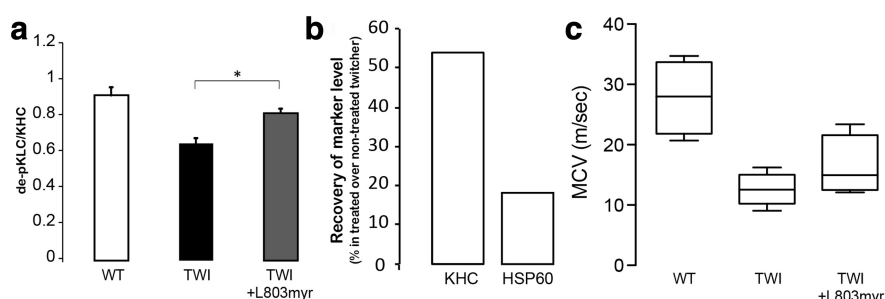


Figure 6. *In vivo* neuroprotection using GSK3 β inhibitors. (**a**) Twitcher mice treated with the myristoylated L803 showed a significant normalization of dephosphorylated KLC levels, indicating a recovery in KLC function (**a**; $p = 0.01$, unpaired t test) as well as in levels of KHC and HSP60 in proximal sciatic nerve stumps (**b**; $p = 0.01$, unpaired t test). Data are expressed as percentage of protein marker accumulated in ligated nerves from treated mutant versus that in nontreated mutants. **c**, Motor conduction velocities (MCV, in meters per second) were recorded on the sciatic nerves of twitcher (Twi) mice treated with L803 and in untreated mutants and wild-type mice of matching age ($n = 4$ mice per group). Although MCV in treated mutants showed a mild improvement, this was not significant.

the microtubular cytoskeleton and its regulatory proteins. For example, mutations or changes in the phosphorylation of the microtubule-binding protein Tau are known to significantly inhibit axonal transport (Stoothoff et al., 2009; Kanaan et al., 2011; Bull et al., 2012; Shahpasand et al., 2012). Because Tau is also a target of PP1 and GSK3 β , it is possible that changes in Tau phosphorylation might indirectly affect the rates of transport in both directions (Bull et al., 2012). Although the recovery of transport using PP1/GSK3 β inhibitors was significant, it was not complete, suggesting the involvement of other regulatory components in psychosine pathogenesis.

How neuronal vulnerability is regulated is still unclear. Previous experiments showed that PP1 is not homogeneously activated

in twitcher axons (Cantuti-Castelvetri et al., 2012), suggesting that the PP1-GSK3 β pathway may be differentially affected in subsets of axons. Selective damage of neurons is observed in other neurological diseases such as Parkinson's and Alzheimer's diseases. The mechanism regulating neuronal vulnerability in these diseases and in Krabbe disease may depend on the occurrence of various pathogenic factors concomitantly in the same cell. In Krabbe disease, this may mean that there is differential accumulation of psychosine in subsets of neurons or that other pathogenic/protective pathways may exist in specific neurons.

Krabbe disease has remained incurable to date. The only traditional treatment providing some improvement (albeit not a cure) is hematogenous replacement (Yeager et al., 1984; Escobar et al., 2005). Our study indicates that pharmacological intervention to protect FAT may be beneficial. We and others (McGraw et al., 2005; Hawkins-Salsbury et al., 2011) have hypothesized that simultaneous engagement of various pathogenic mechanisms limits hematogenous replacement efficacy. Because the pharmacological treatments used in this study were not combined with any metabolic correction, treated mutants were not expected to avoid developing neurological symptoms of the disease. However, the normalization of phosphorylated KLC, the amelioration of FAT, and the mild (albeit not significant) improvement in nerve conduction in twitcher mice treated with GSK3 β inhibitors underline the potential benefit of such neuroprotective interventions. The potential synergy of combining these neuroprotective treatments with hematogenous replacement is currently being evaluated in our laboratory.

In conclusion, our data demonstrate the occurrence of FAT defects in cellular and animal models of Krabbe disease and identify the sphingolipid psychosine as a FAT inhibitor. Elevated psychosine inhibits FAT by activation of the PP1-GSK3 β pathway. Preliminary data on postmortem human brain samples indicate that similar pathogenic activation of GSK3 β occurs in the nervous system of Krabbe patients, underlining the relevance of this axonal defect in children with this disease. Neuroprotection experiments have the potential of treating Krabbe disease neurodegeneration by reversing FAT defects, opening new and promising areas of clinical research relevant for Krabbe disease and other leukodystrophies.

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