Galactosylsphingosine (psychosine) induced demyelination is attenuated by sphingosine 1-phosphate signalling.

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ABSTRACT
Globoid cell leukodystrophy (Krabbe disease, KD) is a rare infantile neurodegenerative disorder. KD is caused by deficiency in the lysosomal enzyme galactocerebrosidase (GALC) resulting in brain accumulation, in the micromolar range, of the toxic metabolite galactosylsphingosine (psychosine). Here we find psychosine induces human astrocyte cell death likely via an apoptotic process in a concentration- and time-dependent manner (EC50 ~15μM at 4h). We show these effects of psychosine are attenuated by pre-treatment with the sphingosine 1-phosphate receptor agonist pFTY720 (Fingolimod) (IC50 ~100nM). Psychosine (1μM, 10μM) also potentiates LPS-induced (EC50 ~100ng/ml) production of pro-inflammatory cytokines in mouse astrocytes, which is also attenuated by pFTY720 (1μM). Most notably, for the first time, we show that psychosine, at a concentration found in the brains of patients with KD (EC50 ~100nM) directly induces demyelination in mouse organotypic cerebellar slices in a manner that is independent of proinflammatory cytokine response and that pFTY720 (0.1nM) significantly inhibits. These results support the idea that psychosine is a pathogenic agent in KD and suggest that sphingosine 1-phosphate signalling could be a potential drug target for this illness.

KEYWORDS
Krabbe disease, astrocytes, demyelination, sphingosine 1-phosphate receptors, pFTY720
INTRODUCTION

Globoid cell leukodystrophy (Krabbe disease, KD) is a rare autosomal recessive neurodegenerative disorder affecting 1:100,000 live births in the United States (Wenger et al., 1997). This lysosomal disorder typically has an early onset, is rapidly progressing and invariably fatal in infants. The vast majority (85-90%) of cases are of the infantile form, with the juvenile and adult onset forms being considered extremely rare (Wenger et al., 1997). The hallmark symptoms of the infantile form include irritability, hypersensitivity, psychomotor arrest and hypertonia. This is followed by rapid mental and motor deterioration, seizures and optic atrophy. Death usually ensues within the first two years of life and there is currently no cure (Davenport et al., 2011). KD is caused by a mutation in the lysosomal enzyme galactosylceramidase (GALC) (Suzuki, 2003). This GALC deficiency results in the accumulation of a toxic lipid metabolite psychosine (galactosylsphingosine) and to a lesser extent, β-galactosylceramide (Giri et al., 2002). Pathological features of KD include profound demyelination and almost complete loss of oligodendrocytes in the white matter, reactive astrocytosis and infiltration of numerous multinucleated macrophages termed ‘globoid cells’. These globoid cells accumulate around blood vessels and in the regions of demyelination and are a unique feature of KD (Suzuki, 2003). Progressive accumulation of psychosine, in the brains of KD patients is thought as the critical pathogenic mechanism of this illness (Davenport et al., 2011). In some cases the levels of psychosine rise more than 100-fold, from sub-nanomolar concentrations to those in the micromolar range (Svennerholm et al., 1980). In KD, the high levels of psychosine escape from lysosomes and dying cells forming aggregates (Orfi et al., 1997). Several reports have demonstrated that psychosine causes direct cellular cytotoxicity by mechanisms that include mitochondrial dysfunction (Haq et al., 2003), caspase activation, alteration of lipid rafts, and modulation of PKC, JNK and NFκB signaling pathways (Davenport et al., 2011; Haq et al., 2003; Yamada et al., 1996). Inflammation is also now accepted to play an important role in the pathogenesis of KD (LeVine and Brown, 1997). Inflammatory molecules, such as AMP-activated protein kinase (AMPK), prostaglandin D, inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines have all been implicated in KD as well as in the twi/twi mouse animal model of this disease (Giri et al., 2008). Taken together, this increasing evidence now suggests that the loss of oligodendrocytes and wide spread demyelination seen in KD is due to apoptotic processes as well as aberrant inflammatory response (Giri et al., 2008; Giri et al., 2006; Haq et al., 2003; Tohyama et al., 2001).

The GALC enzyme, mutated in KD, is involved in the complex pathway of sphingolipid metabolism, which includes bioactive lipids such as ceramide, sphingosine and sphingosine 1-phosphate (S1P), all of them particularly important in regulating neural cell function. In particular, the family of S1P receptors (S1PRs) are G-protein coupled and expressed in a range of cells, including those of the immune, cardiovascular and central nervous systems (Dev et al., 2008; Fyrst and Saba, 2010). These receptors are drug targets for the drug fingolimod, which is the first oral therapy for relapsing remitting multiple sclerosis (MS) (Kappos et al., 2010). The proposed mechanism of action for fingolimod is reported as being dependent on internalisation of S1PRs in T cells limiting their S1P-mediated egress from lymph nodes and thus the attenuation of inflammatory response in the brains of MS patients (Adachi and Chiba, 2008). Importantly, a number of studies have now demonstrated that compounds such as fingolimod can also regulate neuronal and glial cell function (Balatoni et al., 2007; Choi et al., 2011; Fischer et al., 2011; Osinde et al., 2007). Indeed, we and others have shown that S1PRs regulate a number of intracellular signalling pathways in astrocytes and promote astrocyte migration.
(Mullershausen et al., 2007; Mullershausen et al., 2009). In addition, modulation of S1PRs also promotes oligodendrocyte differentiation and survival (Dev et al., 2008; Miron et al., 2008b). S1PRs have also been shown to limit events of demyelination and promote remyelination, which are likely mediated by the dampening of pro-inflammatory cytokine levels (Miron et al., 2010; Sheridan and Dev, 2012). Overall, therefore, S1PRs represent an important drug target that can be exploited for use in neuroinflammatory, demyelinating and neurodegenerative diseases as documented by a growing body of literature (Asle-Rousta et al., 2013; Deogracias et al., 2012). Here we investigate whether regulation of S1P signalling alters psychosine-induced astrocyte dysfunction, pro-inflammatory cytokine release and demyelination.

RESULTS

Psychosine induced human astrocyte cell death is attenuated by pFTY720

The modulation of psychosine on astrocyte cell function is less well studied in KD. We therefore first investigated the effects of this toxin on human astrocyte survival and also demonstrated the protective effects of pFTY720. Cultured human astrocytes were serum starved for 4 h and then pretreated with pFTY720 for 1 h before treatment with psychosine, at the timepoints and concentrations indicated (Fig. 1A). Psychosine reduced human astrocyte numbers in a concentration dependent manner, where psychosine treatment for 4 h significantly reduced astrocyte cell survival (10 µM, 55.4+/−3.8%; 15 µM, 44.3+/−2.7%; and 20 µM, 39.8+/−1.8%, compared to control). Importantly, pre-treatment with 1µM pFTY720 significantly attenuated the psychosine-induced cell death (reduced by 9.8+/−4.1%, 21.1+/−2.5% and 18.4+/−2.2%, respectively) (Fig. 1B). The psychosine (10 µM) induced decrease in survival of human astrocytes was also observed to be time dependent (2 h, 79.2+/−3.2%; 4h, 63.5+/−2.4%; 6 h, 37.1+/−3.3%) and again significantly attenuated in the presence of pFTY720 (reduced by 17.4+/−4.6% at 4 h and 23.1+/−4.3% at 6 h) (Fig. 1D). These effects of pFTY720 were also concentration-dependent, where 1 µM pFTY720 significantly increased cell survival by 34.3+/−3.2%, compared to 10 µM psychosine treatment alone (Fig. 1E). During the course of our experiments demonstrating psychosine-induced astrocyte cell death and reversal by pFTY720 (Fig. 1A-E), we noted these effects were also dependent on the density of cultured astrocytes. To quantify these observations, human astrocytes were seeded at densities ranging from 1.2x10^5 to 6x10^5. The cells were then pre-treated with 1 µM pFTY720 followed by 10 µM psychosine treatment for 2 h and imaged (Fig. 2A). Human astrocytes seeded at low densities of 1.2x10^5 and 2.4x10^5 were most sensitive to psychosine insult, with 81.5+/−6.2% and 68.5+/−5.1% cell death occurring after 2 h treatment. Astrocytes seeded at a density of 3.9x10^5 displayed 39.4+/−5.3% cell death for psychosine treatment alone, which was significantly attenuated to 20.6+/−3.6% in the presence of pFTY720 (Fig. 2B). At the two highest densities, 4.8x10^5 and 6x10^5, 10 µM psychosine treatment for 2 h did not induce overt astrocyte toxicity, with cell densities of 94.4+/−3.7% and 99.9+/−5.4%, respectively (Fig. 2C).
pFTY720 attenuates psychosine-induced decrease of mitochondrial membrane potential in astrocytes

Increasing evidence now suggests the involvement of apoptosis as a mechanism underlying oligodendrocyte cell death seen in KD, where mitochondrial cytochrome c release, alterations in electron transport and loss of mitochondrial membrane potential (ΔΨm) occurs (Haq et al., 2003). Here, the ΔΨm was measured using the membrane-permeant dye tetraethylbenzimidazolylcarbocyanine iodide (JC-1), which exhibits potential-dependent accumulation in mitochondria. At high ΔΨm this dye forms aggregates yielding an emission at 590 nm (red) whereas at low ΔΨm JC1 is primarily in monomeric form yielding an emission at 530 nm (green) (Fig. 3A). Cultured astrocytes were serum starved and pre-treated for 1 h with pFTY720 (1 µM) before treatment with psychosine (5 µM, 10 µM, 15 µM, 20 µM). Cells were then loaded with 1 μM JC-1 and after 30 min the emission spectra was measured. Psychosine treatment of mouse astrocytes decreased the aggregate:monomer ratio of JC-1, indicating loss of ΔΨm compared to control (Fig. 3B). Notably, treatment with pFTY720 attenuated the psychosine-induced decrease in aggregate:monomer ratio of JC-1 returning the ΔΨm close to control levels. These findings were supported by use of the MTT colorimetric cell viability assay (based on the reduction of MTT into formazan crystals by metabolically active cells), which showed psychosine caused a significant concentration-dependent reduction of rat astrocyte viability (10 µM, 73.3 +/-2.7%; 15 µM, 63.5+-/-3.6%; and 20 µM, 49.9+/-1.7%, compared to control). In agreement, pFTY720 significantly attenuated this psychosine induced cell death by 14.4+/-1.7% and 17.3+/-2.5% at 10 µM and 20 µM psychosine concentrations, respectively (Fig. 3C).

Psychosine potentiates lipopolysaccharide (LPS)-induced levels of pro-inflammatory cytokines in mouse astrocytes

The expression of several pro-inflammatory cytokines and chemokines has been observed in the twi/twi mouse model (LeVine and Brown, 1997). Psychosine has also been seen in vitro to markedly potentiate the LPS-induced production of pro-inflammatory cytokines in primary rat astrocyte cultures (Giri et al., 2002). Here, we investigated the effect psychosine on the levels of IL6, TNFα and IL1β in mouse astrocytes in the presence and absence of LPS. Firstly, LPS (1 ng/ml to 10 µg/ml) was shown to induce a concentration dependent increase in the levels of IL6 (Fig. 4A), TNFα (Fig. 4B) and IL1β (data not shown), where 100 ng/mL LPS was selected as the optimal concentration for use in further experiments. Next, cultured mouse astrocytes were serum starved, pre-treated with pFTY720 (1 µM for 1 h) and then treated with LPS (100 ng/mL) and/or psychosine (1 µM and 10 µM) for 3 h, 6 h and 12 h. No discernible release of IL6, TNFα or IL1β was observed after 3 h treatment with LPS and/or psychosine (data not shown). In contrast, the treatment of astrocytes for 6 h with LPS induced at least a 2-fold increase in the levels of IL6 (5.1+/-0.9 pg/ml vs. 31.5+/-4.3 pg/ml) (Fig. 4C), TNFα (19.4+/-3.4 pg/ml vs. 48.3+/-2.1 pg/ml) (Fig. 4D) and IL1β (1.1+/-0.5 pg/ml vs. 14.3+/-5.5 pg/ml) (data not shown), compared to control. Moreover, psychosine (10 µM), while having little effect alone, significantly augmented the LPS induced production of IL6 (31.5+/-4.3 pg/ml vs. 44.8+/-4.2 pg/ml) (Fig. 4C) and TNFα (48.3+/-2.1 pg/ml vs. 59.1+/-2.8 pg/ml) (Fig. 4D) (Unpaired Student’s T test #p<0.05). Importantly, pFTY720 treatment significantly attenuated the increased levels of IL6 (44.8+/-4.2 pg/ml vs. 25.6+/-4.9 pg/ml) (Fig. 4C) and TNFα (59.1+/-2.8 pg/ml vs. 42.4+/-4.4 pg/ml) (Fig. 4D) induced by LPS and/or psychosine (10 µM). Similar findings were observed at 12 h, where pFTY720 attenuated the LPS and/or psychosine (10 µM) mediated increase in
levels of IL6 (132.0+/−3.4 pg/ml vs. 56.4+/−21.3 pg/ml) (Fig. 4E) and TNFα (183.8+/−88.9 pg/ml vs. 78.8+/−25.3 pg/ml) (Fig. 4F). Collectively, these findings suggest that psychosine may enhance the LPS-induced levels of pro-inflammatory cytokines in astrocytes as previously reported (Giri et al., 2002), and that pFTY720 attenuates these effects.

**pFTY720 inhibits psychosine induced demyelination in organotypic cerebellar slices**

Rapid and complete loss of myelin and the myelin forming oligodendrocytes is one of the main pathological features of KD (Davenport et al., 2011). Of interest, pFTY720 promotes remyelination as well as limit demyelination induced by the bioactive lipid lysolecithin (lysophosphatidylcholine, LPC) (Miron et al., 2010; Sheridan and Dev, 2012). With this in mind, we first determined if psychosine induce demyelination in cerebellar slices and secondly examined if pFTY720 attenuate this psychosine induced demyelination. Organotypic cerebellar slices were exposed to LPC (0.5 mg/ml) or psychosine (100 nM, 1 µM, 20 µM) in the presence or absence of pFTY720 (0.1 nM, 1nM) for 18 h and treated for a further 30 h with pFTY720 (0.1nM, 1nM) (Fig. 5A). In agreement with our previous studies (Pritchard et al., 2014; Sheridan and Dev, 2012), pFTY720 attenuated LPC induced demyelination compared with control, as observed by expression of myelin oligodendrocyte glycoprotein (MOG) (Fig. 5B) and myelin basic protein (Fig. 5C-D). Importantly, the exposure of the slice cultures to psychosine also decreased the expression of MOG (Fig. 5B, Supplemental Fig. 1), myelin basic protein (MBP) (33.4+/−3.8 vs 12.4+/−2.5, 1 µM psychosine) (Fig. 5C-D), and myelin proteolipid protein (PLP) (Supplemental Fig. 2), as well as decreasing the expression of neurofilament H (NFH) (29.9+/−5.4 vs 13.4+/−4.6, 1µM psychosine). It is noteworthy, that pFTY720 (0.1 nM, 1nM) prevented psychosine-induced decrease in expression of MOG (Fig. 5B, Supplemental Fig. 1), MBP (12.43+/−2.5 vs 31.6+/−4.9) and PLP (Supplemental Fig. 2), in addition to NFH (13.4+/−4.6 vs 40.7+/−10.3) (Fig. 5C-D). Taken together, therefore, these studies demonstrate that pFTY720 reverses psychosine-induced demyelination and neuronal toxicity in cerebellar slice cultures.

**Psychosine induced demyelination in cerebellar slices occurs independently of pro-inflammatory cytokines**

The involvement of pro-inflammatory cytokines in the pathogenesis of demyelination has been previously investigated (di Penta et al., 2013) and a model where S1PR activation may reduce demyelination via a mechanism involving attenuation of cytokine/chemokine release has been proposed (Sheridan and Dev, 2012). Hence we investigated whether psychosine treatment would induce the release of the pro-inflammatory cytokines IL6, TNFα and IL1β from organotypic cerebellar slices. As with the above protocol, organotypic cerebellar slices were exposed to LPC (0.5 mg/ml) or psychosine (100 nM) in the presence or absence of pFTY720 (0.1 nM) for 18 h and treated for a further 30 h with pFTY720 (0.1 nM). After the 30 h incubation the media was collected and analysed by ELISA. LPC induced at least a 4-fold increase of IL6 (136.2+/−61.5 pg/ml vs. 1543.2+/−89.4 pg/ml), TNFα (15.0+/−4.6 pg/ml vs. 61.9+/−12.3 pg/ml) and IL1β (0.6+/−0.6 pg/ml vs. 178.8+/−32.7 pg/ml), compared with controls (Fig. 6A-C). Notably pFTY720 treatment attenuated the LPC induced release of IL-6 (1543.2+/−89.4 pg/ml vs. 1273.1+/−22.1 pg/ml) (Fig. 6A), TNFα (61.9+/−12.3 pg/ml vs. 42.32+/−11.2 pg/ml) and IL1β (178.8+/−32.7 pg/ml vs. 14.0+/−14.1 pg/ml) (Fig. 7C). Interestingly, psychosine (100 nM) treatment did not induce the release of IL6, TNFα or IL1β, in agreement with our data showing the
treatment of mouse astrocytes with psychosine alone had little effect on the release of IL6 (Fig. 4C and E), TNFα (Fig. 4B and F) and IL1β (data not shown). Furthermore, we did not observe significant effects of psychosine on Ionized calcium binding adaptor molecule 1 (Iba1) (microglia) staining nor did BV2 microglia cells treated with psychosine show enhanced levels of IL6 cytokine release (Supplemental Fig. 3). Taken together, these results suggest that, in organotypic cerebellar slices, psychosine-induced demyelination occurs via a mechanism that is likely independent from the release of pro-inflammatory cytokines. Moreover, it is important that pFTY720 inhibits this type of demyelination that appears to have a mode of action independent of these conventional pro-inflammatory cytokines.

DISCUSSION

Accumulation of psychosine, propagation of pro-inflammatory cytokines, demyelination and the widespread loss of oligodendrocytes are all hallmarks of the KD brain (Suzuki, 1998; Wenger et al., 2001). To date, little is known about the role of astrocytes in KD, where the majority of studies have mainly been focused on the role oligodendrocytes in this illness. Altered astrocytic function has gained recognition as a major contributing factor to a growing number of neurological disorders (Claycomb et al., 2013) and the belief that astrocytic dysfunction significantly contributes to the development of inflammation in the CNS has gained traction in the past number of years (Sharma et al., 2010). In addition, the immunomodulatory functions of astrocytes are now being shown to actively participate in the pathogenesis of a number of demyelinating disorders (Sharma et al., 2010). It has also been reported that astrocytic processes may closely surround demyelinating fibers and that astrocytes release factors such leukemia inhibitory factor (LIF) to promote myelination. Remarkably, when oligodendrocytes from twi/twi mice (twi-oligos) are transplanted into the shiverer mouse model of demyelination, these twi-oligos are capable of myelinating the shiverer axons (Kondo et al., 2005). This indicates that demyelination in KD may not be solely attributed to oligodendrocyte dysfunction and that given correct environmental support these twi-oligos may function normally. Thus, astrocytic reactivity in KD may not represent a secondary response to demyelination, but may possibly be a primary response to psychosine and in turn astrocytes may significantly contribute to the pathogenesis of KD (Claycomb et al., 2013). We therefore, first, included data that demonstrated a direct effect of psychosine on astrocyte cell death and attenuation by pFTY720. Here we investigated the effect of the cytotoxic lipid metabolite psychosine on cultures of human astrocytes. Psychosine caused a time- and concentration-dependent decrease in astrocyte cell numbers as previously reported (Giri et al., 2002; Sugama et al., 1990). In agreement with the current literature (Davenport et al., 2011; Haq et al., 2003; Jatana et al., 2002), this astrocytic cell death induced by psychosine appeared to occur via an apoptotic process as suggested by our JC-1 studies, which showed psychosine induces mitochondrial dysfunction. Importantly, pFTY720 attenuated psychosine-induced cell death as well as restoring mitochondrial dysfunction and increasing cell viability caused by psychosine. Moreover, we found that while psychosine itself did not induce increased levels of pro-inflammatory cytokines in mouse astrocytes, it did enhanced LPS-mediated release of IL6, TNFα and IL1β, and these effects were again reduced by pFTY720. We also report here, for the first time, that direct application of psychosine to organotypic slice cultures induces demyelination in a manner that does not include enhanced pro-inflammatory cytokine release. These data corroborate the idea that psychosine in the brains of KD patients may directly induce demyelination. In these set
of experiments, pFTY720 attenuated LPC induced demyelination which was shown to include enhanced levels of pro-inflammatory cytokines as we have reported before (Sheridan and Dev, 2012). Of most interest, pFTY720 also reduced the demyelination caused by psychosine, in a manner that did not include enhanced levels of IL6, TNFα and IL1β. Overall, these studies suggest that S1PRs may regulate myelination state in both inflammatory and non-inflammatory paradigms.

Inflammatory processes have been implicated in the pathogenesis of KD and the expression of pro-inflammatory cytokines have been reported in the twi/twi mouse brain (Claycomb et al., 2013; LeVine and Brown, 1997). However, the mechanism governing psychosine-mediated cell toxicity and the direct role of pro-inflammatory cytokines in the degeneration of astrocytes and/or oligodendrocytes is still not fully understood. In the present study we found psychosine treatment itself did not alter pro-inflammatory cytokine levels, and as such is unlikely to explain the decrease in astrocyte cell numbers we observed after psychosine treatment. Instead, psychosine may induce astrocyte cell death by altering mitochondrial function and electron transport as determined by increased JC1 levels in the cytosol and decreased NAD(P)H oxidase function measured by MTT, respectively. In agreement with this idea, previous studies have demonstrated that psychosine alters mitochondrial function and electron transfer, likely via a mechanism involving changes in the lipid environment of the membrane (Cooper et al., 1993; Tapasi, 1998). Moreover, studies have also reported that pFTY720 can stabilise mitochondrial function, supporting our findings that pFTY720 rescues mitochondrial dysfunction induced by psychosine. Interestingly, in this current study, while psychosine alone had no effect on pro-inflammatory cytokine levels, it augmented the LPS-induced release of IL6 from mouse astrocytes, with a similar trend for the levels of TNFα and IL1β. This finding is comparable to those reported previously where psychosine potentiated LPS-induced production of TNFα, IL6, IL1β and NO in primary rat astrocytes, which in turn was suggested to induce oligodendrocyte cell death (Giri et al., 2002; Giri et al., 2006). These enhanced levels of cytokines were reduced by pFTY720, in agreement with previous studies from our and other groups demonstrating that S1PR’s play a role in regulating the levels of cytokines in a number of immune and glial cells (Choi et al., 2011; Sheridan and Dev, 2012; Wang et al., 2007; Zhang et al., 2008). Thus, in astrocytes, it appears that psychosine directly modulates mitochondrial function to induce cell death, while in parallel enhancing cytokine levels under conditions of LPS-induced inflammation and that pFTY720 can attenuate these effects of psychosine.

Profound demyelination and almost complete loss of oligodendrocytes are two of the major pathological features of KD. The hypothesis that supraphysiologic levels of psychosine kill oligodendrocytes and result in widespread demyelination is now widely accepted. The mechanisms by which psychosine induces demyelination remains unclear at present. Increasing evidence however now suggests that the wide spread demyelination and loss of oligodendrocytes seen in KD and induced by psychosine is due to apoptotic processes, likely via caspase dependent pathways (Zaka and Wenger, 2004; Tohyama et al., 2001; Giri et al., 2006; Giri et al., 2008; Zaka and Wenger, 2004; Haq et al., 2003). Another proposed mechanism by which psychosine induces toxicity involves the molecules preferential accumulation in lipid rafts, associated with regional cholesterol increases and inhibition of protein kinase C (PKC) activity (White et al., 2011; Davenport et al., 2011, Yamada et al., 1996, Hannun and Bell, 1987). Psychosine treatment of oligodendrocytes has also been shown to induce the generation of LPC and arachidonic acid, where inhibition of secreted phospholipase A2
(sPLA2) attenuates psychosine-induced increases in both LPC and arachidonic acid as well as attenuating psychosine-induced cell death (Giri et al., 2006). Furthermore psychosine accumulation has been reported to induce phosphorylation of neurofilament proteins resulting in reduced radial growth of axons in the KD twitcher mouse model and to induce axonal defect and cell death in isolated neuronal cultures (Cantuti-Castelvetri et al., 2012; Castelvetri et al., 2011). Importantly, S1PRs are known to play many roles in the regulation of differentiation, cell survival and apoptosis of oligodendrocytes, astrocytes and microglia (Dev et al., 2008; Miron et al., 2008b). In addition, previous studies have also shown that S1PRs are expressed on Schwann cells and that treatment with compounds such as pFTY720 can promote Schwann cell survival and regulate peripheral nerve myelination (Kim et al., 2009). Moreover studies have also shown that compounds such as pFTY720 can significantly improve motor function recovery in animal models of spinal cord injury (Lee et al., 2009). Therefore, we utilised organotypic slice cultures as a more complex cellular model to investigate the effect of psychosine on demyelination. Here, for the first time, we showed that psychosine can directly induce a concentration dependent demyelination, as expressed by a decrease in the levels of MOG, PLP and MBP and that pFTY720 can attenuate these effects. These findings are in agreement with our and other previous reports, demonstrating that pFTY720 rescue myelination state (Coelho et al., 2007; Jung et al., 2007; Mattes et al., 2010; Miron et al., 2008a; Miron et al., 2008b; Sheridan and Dev, 2012). Interestingly, unlike LPC, psychosine treatment alone did not induce the release of IL6, TNFα or IL1β from cerebellar slices, which was in agreement with our astrocyte data. These findings suggest that psychosine induces demyelination independent from the release of pro-inflammatory cytokines and most importantly that pFTY720 can inhibit demyelination independent from the regulation of pro-inflammatory cytokines. Taking these current findings and previous studies into account that have demonstrated pFTY720 can promote neuronal and oligodendrocyte survival (Dev et al 2008; Miron et al 2008b), we suggest that pFTY720 may rescue the toxic effects induced by psychosine by having multimodal effects on both glial and neuronal cells. In view of not overstating these findings, however, we acknowledge the need for further studies including: (1) conducting EM analysis that provides ultrastructural evidence of remyelination and thus supports our immunostaining data, which is not a direct measurement of enhanced myelination per se, (2) testing the effects of pFTY720 on myelination in twi/twi cerebellar slice cultures to support the pyschosine-induced demyelination studies and (3) most importantly, testing the effects of pFTY720 on disease progression in twi/twi mouse model. While these studies remain outstanding and while recent clinical data shows a lack of efficacy for pFTY720 in progressive forms of multiple sclerosis, the cellular data presented in the current study goes some way in suggesting that S1PRs may be useful targets in demyelinating illnesses such as KD.
METHODS AND MATERIALS

Astrocyte and Cerebellar Slice Cultures
Human, rat and mouse astrocytes were cultured as we have described before (Healy et al., 2013; Rutkowska et al., 2015). BV2 microglia were cultured in DMEM supplemented with 2% FBS (Labtech) and 1% penicillin/streptomycin (Sigma) at 37°C and 5% CO₂. When 80% confluent cells were split into 24 well plates to be treated. In all cases, before treatments cells were serum starved for 3-4 h by incubating in serum-free DMEM/F12 (Fisher) at 37°C and 5% CO₂. Specific treatment details are indicated in the figure legends. Organotypic cerebellar slice cultures were prepared exactly as we have described previously (Pritchard et al., 2014; Sheridan and Dev, 2012). In brief, tissue isolated from postnatal day 10 (P10) C57BL/6 mice and 400 µm parasagittal slices of cerebellum were grown on cell culture inserts (5–6 slices each) (Millicell PICMORG50 Millipore). Slices were cultured using an interface method with 1 ml of medium per 35 mm well. For the first 3 days in vitro (DIV), slices were grown in serum-based medium (50% Opti-Mem (Invitrogen), 25% Hanks' buffered salt solution (HBSS) (Gibco), 25% heat-inactivated horse serum and supplemented with 2 mM Glutamax, 28 mM D-glucose, 100 U/mL penicillin/streptomycin (Sigma) and 25 mM HEPES, (Sigma) at 35.5°C and 5% CO₂. After 3 DIV, slices were transferred to serum-free medium (98% Neurobasal-A and 2% B-27 (Invitrogen), supplemented with 2 mM Glutamax, 28 mM D-glucose, 100 U/mL penicillin/streptomycin and 25 mM HEPES). Demyelination was induced at 12 DIV and examined at 14 DIV. All tissue was isolated in accordance with EU guidelines and protocols approved by the Trinity College Dublin ethics committee.

Biochemical Analysis
For cytokine analysis supernatants from cell culture were removed and examined for their cytokine content using ELISA kits for IL6 (DY406) TNFα (DY410) and IL-1β (DY401) according to the manufacturer's instructions (R&D systems), and exactly as we have described before (Pritchard et al., 2014). For Western blotting, cerebellar slices were scraped from the culture membrane and suspended in PTXE buffer (PBS, 1% Triton-x, 1 mM EDTA) using mechanical homogenisation and sonication. Samples were denatured and electrophoresis carried out on 10% SDS-polyacrylamide gels exactly as we have previously reported (Pritchard et al., 2014). Primary antibodies used were: anti-MOG (Millipore: MAB5680) and anti-tubulinβ (Millipore MAB3408). Secondary antibody used was: HRP conjugated mouse (Sigma: A8924).

JC-1 and MTT Assays
JC-1 (tetraethylbenzimidazolylcarbocyanine iodide) exhibits potential-dependent accumulation in mitochondria. Therefore measurements of mitochondrial membrane potential (ΔΨm) using membrane-permeant dyes such as JC-1 are widely used in apoptosis studies to monitor mitochondrial health. Mouse astrocytes were plated into a black 96-well plate in 100 µl DMEM/F12 and incubated for 72 h at 37°C and 5% CO₂. On the day of the assay, the cells were incubated for 3 h in serum-free DMEM/F12 at 37°C and 5% CO₂ before receiving a pre-treatment of pFTY720 (1 µM) for 1 h. Cells were then treated with psychosine (5 µM, 10 µM, 15 µM, 20 µM) for 2 h. All wells were then loaded with 1 µM JC-1 for 30 min. After this incubation the plate was centrifuged for 5 min at 400 x g at room temperature. The supernatant was aspirated and the wells washed with 200 µl of PBS. This wash step was repeated and the emission spectra at 535 nm and 590 nm was measured (SpectraMAX Gemini
A colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability as per manufacturer’s instructions (MTT, Invitrogen, M6494), similar to our previous studies (Mullershausen et al., 2009; Rutkowska et al., 2015).

**Light and Fluorescence Microscopy**

All images of human astrocytes were taken using a CKX41 Olympus inverted microscope (Mason Technologies) at 10x magnification. ImageJ software was used to calculate the cell density of the surviving cells by measuring the percentage of empty space compared to their own controls. For organotypic cerebellar slice cultures, immunostaining was performed as we have described previously (Sheridan and Dev, 2012). Primary antibodies used were: anti-MBP (Abcam: ab40390), anti- NFH (Millipore: MAB5539), anti-PLP (Millipore: MAB388), anti-MOG (Millipore: MAB5680) and anti-Iba1 (Wako: 019-1974). Secondary antibodies used were: anti-chicken 633 (Invitrogen Alexa: A21103), anti-rabbit 488 (Invitrogen Alexa: A27034) and anti-mouse Dylight 549 (Jackson ImmunoResearch: 715-505-020). Confocal images were captured using a LSM 510 Meta microscope at 10x or 20x magnification. These resulting images were analyzed using ImageJ software. A total number of 5-6 slices were used per condition and the fluorescence of each cerebellar slice was captured using 5-6 independent regions of interest (ROI). The ROI were selected randomly to cover the whole slice and the mean fluorescence was calculated using a total of 25-36 independent ROI observations, for each independent experiment.

**Statistical Analysis**

All statistical analysis was performed using Prism 5 GraphPad Software package. A one-way ANOVA with Newmann-Keuls post-hoc test was used to compare groups and an unpaired Students T-test was used to compare two sets of data to each other. Individual statistical tests are described in text and figure legends. The significance levels (or alpha levels) were set at p<0.05*, p<0.01** and p<0.001***.
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Competing interests
The authors declare no competing interest for this study.

Author contributions
K.K.D. conceived, supervised and co-ordinated the study. C.O.S. performed the experiments and analysed the data. K.K.D. and C.O.S. wrote the manuscript.

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Fig. 1: pFTY720 attenuates psychosine-mediated astrocyte cell death.

(A) Diagram of experimental timeline and treatments. Human astrocytes were pre-treated with 1 µM pFTY720 (pFTY) for 1 h followed by 5 µM, 10 µM, 15 µM and 20 µM psychosine (Psy) for 1-6 h. Cells were imaged under light microscopy. (B) Concentration-dependent psychosine induced cell death is attenuated by pFTY. Human astrocytes were treated with 5 µM, 10 µM, 15 µM and 20 µM psychosine for 4 h +/- pFTY. (C) Time-dependent psychosine induced cell death is attenuated by pFTY. Human astrocytes were pre-treated with 1 µM pFTY for 1 h followed by 10µM psychosine for 1, 2, 4 and 6 h. (D) pFTY attenuates psychosine-induced astrocyte cell death in a concentration-dependent manner. Human astrocytes were pre-treated with 10 nM, 100 nM and 1 µM pFTY for 1 h followed by 10 µM psychosine for 2 h. In all cases, image analysis was performed using Image J software and graphical data is presented as mean +/- SEM (n=3-6). Representative images are also shown. Statistical analysis was performed using one-way ANOVA and Newman-Keuls multiple comparison post-test *p < 0.05, **p < 0.01 compared to pFTY; ## p <0.01, ### p <0.001 compared to control.
Fig. 2: Psychosine induced cell toxicity is dependent on astrocyte cell density.

(A) Human astrocytes were seeded at densities ranging from 1.2x10⁵ to 6x10⁵, pre-treated with 1 µM pFTY for 1 h followed by 10 µM psychosine for 2 h. (B) Image analysis was performed using Image J software. Data presented as mean +/- SEM (n=4), one-way ANOVA and Newman-Keuls multiple comparison post-test *** p < 0.001. (C) Representative images showing psychosine induced cell loss, with or without pFTY treatment.
Fig. 3. pFTY720 attenuates psychosine-induced decrease of mitochondrial membrane potential in astrocytes.

(A) Schematic shows that at high mitochondrial membrane potentials JC-1 forms red aggregates (590 nm) in mitochondria while at low mitochondrial membrane potentials the JC-1 is predominately found as a green monomer (535 nm) in the cytosol. (B) Treatment of mouse astrocytes with psychosine (2 h) resulted in a decrease in the ratio of red aggregates to green monomers to ~75% of controls, while pre-treatment with pFTY (1 µM, 1 h before addition of psychosine) attenuated this decrease. Data presented as mean +/- SEM (n=3), unpaired student t-test *p < 0.05, ** p < 0.01 comparing psychosine +/- pFTY. (C) Graph shows MTT assays performed on rat astrocytes treated with psychosine for 2 h +/- pFTY. MTT absorbance read at 540 nm. Data presented as mean +/- SEM (n=3), one-way ANOVA and Newman-Keuls multiple comparison post-test *p < 0.05, ** p < 0.01 comparing psychosine +/- pFTY; ## p <0.01, ### p <0.001 comparing control +/- psychosine.
Fig. 4. Psychosine potentiates LPS-induced production of proinflammatory cytokines in primary mouse astrocytes.

Mouse astrocytes were serum starved for 4 h and pre-treated for 1 h with 1 µM pFTY before treatment with LPS and/or 1 µM or 10 µM psychosine. The supernatant was collected and an ELISA performed. Treatment of mouse astrocytes with LPS (1 ng/ml - 10 µg/ml) induced a concentration-dependent increase in levels (A) IL6, (B) TNFα. Mouse astrocytes treated with 100 ng/ml LPS +/- psychosine treatments for (C,D) 6 h or (E,F) 12 h showed an increase in (C,E) IL6, (D,F) TNFα, that was attenuated by pre-treatment with 1 µM pFTY. Data is presented as mean +/- SEM (n=3-4), one-way ANOVA and Newman-Keuls multiple comparison post-test *p < 0.05, *** p < 0.001; unpaired student t-test # p<0.05 compared to LPS alone.
Fig. 5. pFTY720 treatment inhibits psychosine induced demyelination of cerebellar slices.

(A) Organotypic slice cultures were prepared from the cerebellum of P10 mice and grown in culture for 12 days. Slices were treated with LPC, psychosine and/or pFTY for 18 h. The media was then changed and pFTY treatment continued for a further 30 h. Cerebellar cultures were then processed for Western blotting or immunocytochemistry. (B) LPC (0.4 mg/ml) and psychosine (100 nM or 1 μM) treatment induced a reduction in MOG expression, which was rescued by pFTY720 (0.1 nM) treatment. (C) Representative confocal images displaying MBP (MBP, green) and neurofilament (NFH, red) immunostaining under treatment conditions indicated. Confocal images captured at ×10 magnification. Treatment with pFTY (0.1nM) attenuates LPC (0.4 mg/ml) and psychosine (100 nM or 1 μM) induced demyelination. ML (molecular layer), WM (white matter), PCL (Purkinje cell layer) and GCL (granule cell layer). (D) Bar graph illustrates changes in MBP and NFH staining after LPC (0.4 mg/ml) and psychosine (100 nM, 1 μM) +/- pFTY (0.1 nM) treatments. Mean fluorescence was calculated using a total of 25-36 independent ROI observations in each experiment. Data presented as mean +/- SEM (n=3), one-way ANOVA and Newman-Keuls multiple comparison post-test *p < 0.05; # p <0.05 comparing control +/- psychosine.
Fig. 6. Psychosine treatment did not induce the release of pro-inflammatory cytokines from cerebellar slice cultures.

Organotypic slice cultures prepared from the cerebellum of P10 mice were grown in culture for 12 days before treatment with LPC (0.4 mg/ml), psychosine (100 nM) and/or pFTY (0.1 nM) for 18 h. Media was then changed and pFTY treatment continued for a further 30 h. The media was collected and cytokine analysis performed by ELISA. Psychosine treatment did not induce the release of (A) IL6, (B) TNFα and (C) IL1β from cerebellar slice cultures. In contrast, treatment with LPC induced the release of all three cytokines analysed, where pFTY attenuated this LPC-induced release of (A) IL6 (n=3), (B) TNFα (n=3) and (C) IL1β (n=2). Data is presented as mean +/- SEM, one-way ANOVA and Newman-Keuls multiple comparison post-test *p < 0.05, ***p < 0.001.
References


