Pathophysiology of Krabbe disease

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Krabbe disease is a rapidly progressive lysosomal storage disorder that primarily affects infants. Accumulation of psychosine, a cytotoxic substrate, in the myelin-forming cells results in death of these cells and myelin degeneration. Myelin debris activates a neuroinflammatory response which plays a significant role in the pathogenesis of this disease. The exact mechanisms by which psychosine causes cell death and activates the pathogenic cascade are unknown. The *twitcher* mouse is an animal model of Krabbe disease that has been extensively utilized for pathophysiological research. There is increasing evidence that the mode of psychosine-mediated cell death is apoptosis. Psychosine has been found to activate secretory phospholipase A_2 in cultured oligodendrocytes. Psychosine has also been reported to up-regulate the stress-activated protein kinase signaling transduction pathway and down-regulate cell survival pathways in cultured oligodendrocytes. A more recent finding is that psychosine induces peroxisomal dysfunction in the *twitcher* brain. This review aims to provide an overview of the current knowledge and understanding of the pathophysiology of Krabbe disease and address the limitations of current treatment strategies.

Keywords: globoid cell leukodystrophy, Krabbe disease, lysosomal storage disorder, oligodendrocyte, psychosine, twitcher

Abbreviations: AA, arachidonic acid; BMT, bone marrow transplantation; CNS, central nervous system; LSD, lysosomal storage disorder; PNS, peripheral nervous system; PPAR-α, peroxisome proliferator-activated receptor alpha; sPLA$_2$, secretory phospholipase A$_2$; TNF-α, tumour necrosis factor-alpha

Introduction

Krabbe disease, or globoid cell leukodystrophy, is a neuroinflammatory lysosomal storage disorder that affects humans, primarily infants, as well as several animal species. The *twitcher* mouse is an authentic animal model of human Krabbe disease that has been utilised extensively for research purposes. Krabbe disease is an autosomal recessive disorder caused by mutations in the gene encoding the lysosomal hydrolase galactosylceramidase. This enzyme breaks down galactosylceramide, a glycolipid found almost exclusively in myelin, and psychosine (galactosylsphingosine), a cytotoxic metabolite of galactosylceramide. Psychosine accumulation is believed to be the primary cause of the rapid degeneration of the myelin-forming cells and consequent demyelination that is seen in this disease (Miyatake and Suzuki, 1972). The myelin-forming cells are the oligodendrocytes and Schwann cells in the central nervous system (CNS) and peripheral nervous system (PNS), respectively.

The mechanisms by which psychosine mediates cell death remain unclear. Further research into these mechanisms is needed to advance understanding and treatment of this fatal disease. This paper will review current knowledge of the pathophysiological mechanisms operating in Krabbe disease, with particular focus on the death of oligodendrocytes in the CNS.

1. Lysosomal storage disorders

Lysosomal storage disorders (LSDs) are a group of inherited progressive diseases that are characterised by the accumulation, or 'storage', of undegraded molecules within the lysosomes of cells. The importance of lysosomes to cell function is shown by the number of different LSDs and their diverse and severe clinical manifestations (see Table 1). There are currently over 50 known LSDs (Ballabio and Gieselmann, 2009); the majority of which are caused by mutations in a single gene that encodes a specific lysosomal hydrolase. However, some LSDs result from a genetic defect in one of the proteins involved in the biogenesis of lysosomal hydrolases.

The cell types in which storage occurs and the body systems affected vary between LSDs. This variation is explained as only those cells that synthesise the substrate of the deficient enzyme or encounter this substrate through endocytosis will be affected (Jeyakumar et al., 2005). Thus the pathology of specific LSDs is often confined to specific cell populations. This explains why some LSDs result in pathology throughout different tissue and organs while the pathology of others, such as Krabbe disease, are confined to the nervous system. With the exception of three X-linked disorders, all LSDs have an autosomal recessive mode of inheritance (Vellodi, 2005). Although individually rare, collectively the prevalence of LSDs in humans in Australia is around 1 in 7,700 live births (Meikle et al., 1999; Poorthuis et al., 1999).

Table 1 Clinical features of some of the more common LSDs

LSD	Defective protein	Clinical features
Gaucher disease type I	β-Glucoceramidase	Multi-system disease characterised by hepatosplenomegaly, bone disease and immune dysfunction (Cox, 2001).
Mucopolysaccharidosis (MPS) type I	α-Iduronidase	Multi-system disease characterised by mental retardation, skeletal abnormalities, hepatosplenomegaly, cardiac and respiratory disease (Wraith, 2004).
Metachromatic leukodsystrophy	Arylsulfatase A	Demyelinating disease characterised by motor and mental deterioration (Wraith, 2004).
Fabry disease	α-Galactosidase A	X-linked multi-system disease characterised by reticuloendothelial dysfunction, neurological involvement, cardiomyopathy and renal failure (Desnick et al., 2003).
Krabbe disease	Galactosylceramide	Demyelinating disease characterised by rapid motor and mental deterioration (Suzuki, 2003a).
Pompe disease	α-Glucosidase	Multi-system disease characterised by cardiomyopathy, myopathy and respiratory dysfunction (van den Hout et al., 2003).
Tay-Sachs disease	β-Hexosaminidase A	Neurodegenerative disease characterised by motor and mental deterioration (Wraith, 2004).

LSDs can be classified according to the biochemical nature of the accumulating substrate. For example, the sphingolipidoses are a subgroup of LSDs where there is progressive accumulation of sphingolipids (Platt and Walkley, 2004). Other LSD subgroups include the mucopolysaccharidoses and glycoproteinoses. The majority of LSDs are divided into infantile, juvenile and adult subtypes, depending on the age of disease onset and clinical severity (Meikle et al., 1999). The infantile forms are the most common and also the most severe and rapidly progressing subtype of LSDs, with death usually occurring in the first few years of life (Wraith, 2004). The reasons behind the heterogeneity in disease onset and clinical signs of each LSD are not clear. It may be due to differences in residual enzyme activity, with lower residual activity resulting in earlier accumulation of a large substrate load and clinical signs (Vellodi, 2005). There have been a number of different mutations identified within the same gene for most LSDs (Futerman and van Meer, 2004); certain mutations may be less deleterious to the biological activity of the resulting protein. However, this genotype-phenotype correlation has failed to be proven for most LSDs. Background genetic and environmental factors have also been proposed to contribute to the observed phenotypic diversity (Futerman and van Meer, 2004).

The causes of pathology and clinical signs of LSDs are not only attributable to primary cellular storage but also arise due to complex secondary and tertiary disruptions in cell signalling pathways (Vellodi, 2005), which are poorly understood. As the biochemical nature of the accumulating substrate and secondary metabolites generally varies between LSDs, it is reasonable to speculate that the downstream cellular pathways activated should also vary, resulting in pathology that is largely unique to each specific disorder. Despite this, there are many similarities between LSDs. There is evidence that dysfunction of pathways involved in the breakdown of intracellular proteins, the autophagosome-

lysosome and ubiquitin pathways, is universal among these diseases (Bifsha et al., 2007; Settembre et al., 2008). Also, many LSDs have an inflammatory component that plays a role in the disease pathogenesis (Castaneda et al., 2008) and neurological involvement is common (Platt and Walkley, 2004), indicative of the importance of the lysosomal system in the normal function of the nervous system, particularly given there is a lack of cell turn-over.

Delineating the cellular interactions that take place between lysosomal storage and cell dysfunction and death for specific LSDs is both a current and future research endeavour. Progress in this area is likely to lead to more specialised treatments for LSD patients as well as to advance knowledge and understanding of normal cell physiology.

2. Physiological considerations

2.1. The endosomal-lysosomal system

The endosomal-lysosomal system of mammalian cells is involved in degrading, recycling and sorting both intra- and extracellular materials, including damaged cellular machinery, nutrients and foreign substances. The complex system can be compartmentalised into early endosomes, late endosomes and lysosomes, all of which are subcellular membrane-bound organelles (Mukherjee et al., 1997). Material internalised by endocytosis is quickly delivered to early endosomes, the acidic lumen of which promotes dissociation of any receptor-ligand complexes, allowing receptors to be recycled back to the cell surface (Mukherjee et al., 1997). Much of the remaining material cannot be degraded in the early endosomes and so passes through to late endosomes and lysosomes, where final degradation takes place.

There are about 60 lysosomal hydrolases (Pollard and Earnshaw, 2004). Lysosomal prohydrolases are manufactured in the rough endoplasmic reticulum, and then transported to the cis-Golgi apparatus where they are tagged with mannose-6-phosphate moieties, which then bind to mannose-6-phosphate receptors in the trans-Golgi network. These receptors are concentrated in single transmembrane domains so that the prohydrolases can be packaged into transport vesicles which then bud off from the trans-Golgi network. These vesicles deliver the enzymes to late endosomes of the endocytic pathway (Pollard and Earnshaw, 2004). Dissociation of the prohydrolases from their receptors in the late endosome produces activated hydrolases. Fusion between late endosomes and early lysosomes is thought to be the mechanism by which the contents of each organelle are transferred (Mukherjee et al., 1997). Alternatively, release of prohydrolases from mannose-6-phosphate receptors into a vacuolar portion of the late endosome which then develops into a lysosome may occur (Mukherjee et al., 1997).

2.2. Myelin

Myelin is the specialised, biochemically modified plasma membrane processes of oligodendrocytes and Schwann cells. During brain and spinal cord development, when myelin specific genes begin to be expressed, these processes extend and spiral around the axons of nerves to form concentric lamellae (Baumann and Pham-Dinh, 2001). Myelin is composed of 70 – 85% lipid and 15 – 30% protein (dry weight) (Quarles et al., 2006). Galactosylceramide is the most typical myelin lipid making up approximately 20% of the lipid dry weight component (Baumann and Pham-Dinh, 2001; Deber and Reynolds, 1991).

Myelin forms around axons in regular segments, or internodes, such that short lengths of unmyelinated axon (nodes of Ranvier) separate adjacent internodes. This enables rapid saltatory conduction of action potentials (electrical impulses) along myelinated nerve fibres. Membrane depolarisation and thus action potential propagation can only occur at the nodes, where voltage-gated sodium channels are concentrated (Sherwood et al., 2005). The result is that impulses "jump" from node to node. The high lipid content of myelin makes it highly resistant to water-soluble ions which prevents impulse propagation along myelinated parts of the axon (Baumann and Pham-Dinh, 2001). In contrast to myelinated fibres, impulse propagation along unmyelinated fibres is much slower and continuous.

Both myelinated and unmyelinated nerve fibres are present in the CNS and PNS. In general, myelinated fibres have larger axons and innervate tissue which requires signals with greater urgency from the brain, such as skeletal muscle (Sherwood et al., 2005). Fibres which relay information to the

brain from proprioceptors in muscles, tendons and ligaments are also myelinated, enabling voluntary control of proprioception (Brodal, 2004). Schwann cells are intimately associated with the axon segment they myelinate. There is one Schwann cell for each internode in the PNS (Sherman and Brophy, 2005). In contrast, one oligodendrocyte can extend many processes and myelinate up to 30 to 40 internodes in the CNS, which may be on different axons (Sherman and Brophy, 2005). Thus, the death of one oligodendrocyte can result in the dysfunction and conduction delay of more than one nerve.

Knowledge of the normal anatomical and biochemical formation of myelin is important in understanding paediatric white matter diseases such as Krabbe disease. Myelin formation in the human CNS begins in the spinal cord at 12 to 14 weeks gestation (Weidenheim et al., 1992) and continues well into adulthood in the cerebral cortex (Sampaio and Truwit, 2001). However, the most critical and rapid period of myelin formation, and thus oligodendrocyte proliferation and maturation, occurs between mid-gestation and 2 years of age (Kinney et al., 1994). Contrary to early beliefs, the structure of myelin is dynamic (DeWille and Horrocks, 1992); however, there is limited knowledge concerning the rates of metabolic turnover of specific myelin constituents (Baumann and Pham-Dinh, 2001). Generally, the turnover of these constituents is multiphasic, with faster rates occurring earlier in life followed by much slower rates once myelination is complete (Quarles et al., 2006). The accumulation of psychosine in the brains of Krabbe patients and *twitcher* mice indicates that significant galactosylceramide turnover occurs during early myelination and myelin remodelling.

The temporal and spatial anatomical pattern of myelination in the developing human brain has been well studied by magnetic resonance imaging (Paus et al., 2001). In general, myelination proceeds in a posterior to rostral direction (Takeda et al., 1997) (from spinal cord to occipital lobes to frontal lobes) and medial to lateral within the white matter (Rice and Barone, 2000). Less work has been done on the temporal and spatial biochemical pattern of myelin development in the human CNS. Kinney et al. (1994) found that irrespective of site, phospholipids and cholesterol are expressed first in the white matter, followed by myelin-associated lipids and proteins; firstly sphingomyelin, then cerebrosides (including galactosylceramide, the substrate of the deficient enzyme in Krabbe disease) simultaneously with myelin basic protein and proteolipid protein, and finally sulfatides. Galactosylceramide was found to be present at birth in the posterior limb of the internal capsule but did not appear until approximately 4 and 7 months of age in the corpus callosum and frontal lobes, respectively (Kinney et al., 1994). This suggests the pattern of pathology predicted in Krabbe disease.

When conducting research on animal models of human white matter diseases, such as the *twitcher* mouse, it is also important to consider the normal pattern of myelin formation in that specific animal. There is no robust relationship between brain development in mice and humans (Watson et al., 2006). However, parallel stages of myelination have been drawn. While the most rapid period of myelination in the human CNS occurs between mid-gestation and 2 years of age, the most rapid stage in the mouse CNS occurs between postnatal day 10 and 30 (Barbarese et al., 1978; Morell et al., 1972). Myelin in the corpus callosum of the mouse has been first detected at postnatal day 14 (Vincze et al., 2008). In humans, myelin in the corpus callosum is first detected around 1 to 3 months postnatal (Paus et al., 2001). Despite the altricial commonality, there is much evidence that brain development in general is less advanced at birth in mice and rats compared with humans (Romijn et al., 1991) and that, unlike in humans, little myelination occurs prenatally (Wiggins, 1982).

3. Krabbe disease in humans

Krabbe disease is named after the Danish physiologist Knud Krabbe who first reported the disease in humans as a 'familial infantile form of diffuse brain sclerosis' in 1916 (Krabbe, 1916). The disease affects around 1 in 100,000 live births in the United States (Wenger et al., 1997). In the Australian population, the prevalence has been estimated to be 0.74 in 100,000 (Meikle et al., 1999), while in the Netherlands a prevalence of 1.35 in 100,000 has been reported (Poorthuis et al., 1999). Most cases of Krabbe disease are of the infantile form although juvenile and adult-onset forms do exist (Kolodny et al., 1991). Clinical signs of infantile Krabbe disease have been roughly divided into three stages (Hagberg et al., 1963). The first stage emerges between 3 and 6 months of age and is characterised by irritability, hypersensitivity, fever and limb stiffness (Suzuki, 2003a). The second stage is marked by rapid motor and mental deterioration, generalised hypertonicity and optic atrophy (Suzuki, 2003a). In the final stage the infant regresses into a decerebrate condition (Wenger et al., 2000) and death occurs before the age of two.

4. The *twitcher* mouse

The *twitcher* mouse is an enzymatically authentic model of human Krabbe disease (Kobayashi et al., 1980). The galactosylceramide mutation arose spontaneously in the mouse and was first observed in the CE/J strain in 1976 at the Jackson Laboratory (Bar Harbor, Maine). The mutation is inherited in an autosomal recessive fashion as in the human. Due to poor breeding traits of CE/J mice the mutant allele was transferred to the C57BL/6J strain (Duchen et al., 1980) and has since been maintained by heterozygote crosses for research purposes. The discovery of a nonsense mutation in the murine galactosylceramidase gene (Sakai et al., 1996) has enabled the development of a polymerase chain reaction based test which is used to identify heterozygote (carrier) and recessive homozygote (*twitcher*) animals immediately after birth. Spontaneous animal models of human Krabbe disease have also been described in other species, including the dog (Wenger et al., 1999) and rhesus monkey (Baskin et al., 1998), although the *twitcher* mouse remains the most extensively investigated model and an invaluable research tool, particularly given the rarity of the disease in humans.

The clinico-pathological features of the *twitcher* are similar to those observed in Krabbe patients. *Twitcher* mice appear normal from birth until around 20 days of age, after which clinical signs begin to emerge. Failure to gain weight, inactivity and a generalised tremor are initial signs (Duchen et al., 1980; Suzuki and Taniike, 1995). This is followed by a hunched posture, progressive muscular weakness, particularly in the hind limbs, and hind limb paralysis (Duchen et al., 1980). *Twitchers* rarely survive beyond 45 days of age (Suzuki and Taniike, 1995). Clinical signs coincide with the severity of demyelination, which can be detected in the PNS as early as day 10 and in the spinal cord by day 20 (Suzuki and Taniike, 1995). Thereafter demyelination commences in the cerebellum and cerebrum, progressing rapidly until death. An inflammatory macrophage infiltrate can be detected in the PNS white matter at day 5 (Tanaka et al., 1988) and throughout the CNS white matter by day 20 (Ohno et al., 1993b). Microgliosis and astocytosis can be observed from day 20 in the CNS (Suzuki and Taniike, 1995). The phagocytosis of myelin debris by microglia/macrophages results in the formation of globoid cells, the pathological hallmark of the disease.

There are some discrepancies between the disease in *twitcher* mice and human Krabbe patients. In contrast to humans, *twitchers* do not exhibit seizures or decerebrate rigidity (Suzuki and Taniike, 1995). The PNS is more severely affected in the *twitcher* (Duchen et al., 1980) while brain pathology is milder compared with human Krabbe patients. This is likely a reflection of differences in the relative amount of myelin in these areas (Igisu et al., 1983). While the lipid content of the brain decreases dramatically over the course of the disease in humans, the lipid content in the *twitcher* brain remains close to normal even in the moribund animal (Igisu et al., 1983), which is again indicative of a relatively lower quantity of myelin in the murine brain.

5. Pathophysiology of Krabbe disease

5.1. Psychosine

Psychosine is metabolic by-product in the catabolism of galactosylceramide that is virtually undetectable in the normal brain owing to its rapid breakdown to sphingosine and galactose by galactosylceramidase. Psychosine is synthesised by galactosylation of sphingosine, which is catalysed by the same enzyme which catalyses the synthesis of galactosylceramide (Suzuki, 2003b). This enzyme is only expressed in oligodendrocytes and Schwann cells with its activity being highest during active myelination (Suzuki, 2003b). It is possible that psychosine is produced by the deacylation of galactosylceramide (Miyatake and Suzuki, 1972), although this is still hypothetical (Tohyama et al., 2001). Psychosine has no known function in normal cellular physiology (Suzuki, 2003b).

Evidence for a role played by psychosine in oligodendrocyte degeneration can be seen by comparing the pathogenesis of Krabbe disease to that of the metabolically similar LSD, metachromatic leukodystrophy. In metachromatic leukodystrophy, the enzymatic defect results in an inability to catabolise galactosylsulfatide, a glycolipid which, like galactosylceramide, is enriched in myelin. The build-up of galactosylsulfatide in oligodendrocytes impairs their ability to synthesise myelin and demyelination results (Platt and Walkley, 2004). Compared with Krabbe disease, the clinical onset of metachromatic leukodystrophy is later, disease progression is slower (Wraith, 2004) and there is

significantly less degeneration of oligodendrocytes (Suzuki, 1998). As injection of galactosylceramide into the brain is not detrimental to oligodendrocytes (Suzuki, 2003b), these differences appear to be explained by psychosine.

The cytotoxicity of psychosine has been well demonstrated. When injected into rat brains, psychosine causes severe haemorrhagic infarct, necrosis and myelin degeneration (Suzuki and Tanaka, 1976). Psychosine has been shown to cause death of oligodendrocytes (Cho et al., 1997) and Schwann cells (Tanaka and Webster, 1993) *in vitro*. Whether psychosine-mediated death of myelin-forming cells occurs by apoptosis or necrosis is still not clear. Oligodendrocytes undergoing apoptosis in *twitcher* mice have been detected by TUNEL staining (Taniike et al., 1999). However, this apoptosis may have been induced by proinflammatory cytokines as opposed to psychosine, in particular tumour necrosis factor-alpha (TNF-α), which is secreted from microglia and macrophages that become activated following psychosine-mediated myelin degeneration.

5.1.1. Oligodendrocyte apoptosis

There is increasing evidence that the mode of oligodendrocyte death induced by psychosine is apoptosis. DNA fragmentation, a marker of apoptosis, was detected in MOCH-1 (glial derived) cells and cultured fibroblasts that were incubated with psychosine (Tohyama et al., 2001). Interestingly, the percentage of glial derived cells that underwent apoptosis was significantly higher than that of fibroblasts, which suggests a susceptibility of oligodendrocytes to apoptosis (Tohyama et al., 2001). A study by Haq et al. (2003) used an ApopTag® kit to compare apoptosis in psychosine and vehicle treated MO3.13 oligodendrocyte cells. No apoptotic cells were detected among controls but apoptosis of cells incubated with psychosine was detected. In addition, laddering of DNA isolated from psychosine treated cells was observed, a further indicator of apoptotic cell death (Haq et al., 2003). These results are supported by Zaka and Wenger (2004), who used a TUNEL assay to detect apoptosis of cultured oligodendrocyte progenitor cells incubated with psychosine. The latter study observed that apoptosis occurred in control cells as well (Zaka and Wenger, 2004), a contrast that may be attributed to the difference in cell maturity.

5.1.2. Inhibition of protein kinase C

Psychosine is a potent inhibitor of protein kinase C (PKC) *in vitro* (Hannun and Bell, 1987). PKC is a family of protein kinases that have many biological functions, some of which include regulation of cell growth, regulation of transcription and modification of membrane structure (Newton, 1995). Disruption of the signal transduction pathways involved in these functions could eventually lead to cell death (Hannun and Bell, 1987). A study by Yamada et al. (1996) found that growth and proliferation of *twitcher* Schwann cells in response to a number of growth factors which act through the PKC pathway was reduced compared to normal Schwann cells. This is in agreement with the study by Hannun and Bell (1987) and indicates impairment of PKC signalling by psychosine. However, this inhibition of PKC contrasts somewhat with a more recent study which showed that psychosine mediates the activation of secretory phospholipase A_2 (sPLA$_2$) in the brains of both Krabbe patients and *twitchers* (Giri et al., 2006). Secretory phospholipase A_2 hydrolyses phospholipids to generate fatty acids, which have been shown to enhance activation of PKC (Nishizuka, 1995).

5.1.3. Signal transduction pathways

Cells do not function in isolation; most cell functions occur as a result of interactions with substances derived from the extracellular milieu. Mitogen activated protein kinases (MAPKs) are a superfamily of enzymes that are activated by extracellular stimuli and are responsible for regulating an extensive range of cellular processes, including mitosis, gene expression, metabolism and apoptosis (Roux and Blenis, 2004). There are three major branches, or subfamilies, of the MAPK pathway that have been identified in mammals; the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK) and the p38 protein kinases (Haq et al., 2003). ERKs are generally activated in response to growth factors and other mitogens (Roux and Blenis, 2004) while the JNKs and p38 kinases are preferentially activated by stress stimuli such as proinflammatory cytokines, oxidative stress and UV radiation (Kyriakis and Avruch, 1996).

JNKs have been implicated as a regulator of both intracellular apoptotic and anti-apoptotic signalling pathways depending on the cell type and nature of the extracellular stimuli (Liu and Lin, 2005). Activation of JNK in turn activates c-Jun, a transcription factor which is required for JNK pathway-mediated apoptosis, although the reasons why are still unclear (Lin, 2003). JNKs may induce apoptosis by modulating the pro-apoptotic Bcl-2 (Lei and Davis, 2003), a protein family that can regulate cytochrome c release from mitochondria (Hengartner, 2000). Also, in the absence of nuclear factor kappa-B activation, JNKs have been found to promote TNF-α induced apoptosis (Karin and Lin, 2002).

Nuclear factor kappa-B (NF-κB) are transcription factors that can promote cell survival by inducing the expression of genes which suppress apoptosis (Karin and Lin, 2002). In response to certain stimuli, NF-κB dissociates from its IκBα inhibitor in the cytoplasm and translocates to the nucleus where it binds to target DNA sites (Baldwin, 1996). This activation of NF-κB occurs in response to ligand binding to several cell surface receptors, including TNF receptors and toll-like receptors (Gilmore, 2006).

Treatment of oligodendrocytes with psychosine has been found to reduce both nuclear translocation and DNA binding of NF-κB (Haq et al., 2003). In a study by Haq et al. (2003), cultured oligodendrocyte cells were treated with lipopolysaccharide, an inducer of NF-κB activation (Andreakos et al., 2004), which resulted in increased DNA binding activity of NF-κB. However, the addition of psychosine to lipopolysaccharide treated cells reduced this DNA binding activity and also resulted in a higher concentration of cytosolic IκBα. The same study found that the DNA binding activity of activator protein 1 (AP-1), a downstream product of the JNK signalling cascade, was increased in psychosine treated cells, suggesting that psychosine also up-regulates the JNK signal transduction pathway (Haq et al., 2003). Together, up-regulation of the JNK pathway and down-regulation of NF-κB by psychosine could result in cell apoptosis.

5.1.4. Activation of caspases

The caspases are a family of cysteine proteases of which there are around a dozen that play a central role in apoptosis (Hengartner, 2000). Caspases are synthesised as inactive zymogens, which consist of a prodomain, a small subunit and large subunit, and must undergo activation before they can initiate apoptosis (Hengartner, 2000). There are up-stream, or initiator caspases (e.g. caspase-8, -9 and -10) and down-stream, or effector caspases (caspase-3, -6 and -7) (Earnshaw et al., 1999). Upstream caspases are activated in response to a diverse range of death stimuli, the molecular mechanisms of which are still unclear (Hengartner, 2000). Activated upsteam caspases can activate downstream caspases in what is termed a caspase cascade (Earnshaw et al., 1999). Activated downstream caspases will then cleave and inactivate a select set of cellular proteins that may be involved in protective pathway signalling or cytoskeleton and nuclear structure, among many other functions, causing cell death (Earnshaw et al., 1999).

Psychosine induced apoptosis in cultured cells has been found to involve activation of caspase 9, caspase 8 and caspase 3 (Giri et al., 2008; Giri et al., 2006; Haq et al., 2003; Zaka and Wenger, 2004). Caspase 9 is activated by binding to a protein cofactor, called Apaf-1, in the presence of cytochrome c (Li et al., 1997). The finding that cells incubated with psychosine release cytochrome c from mitochondria is in agreement with this (Haq et al., 2003). However, cytochrome c release from mitochondria can also occur in the late stages of apoptosis, after caspase activation (Hengartner, 2000). Activation of caspase 9 in turn activates caspase 3 (Li et al., 1997) and apoptosis results. Caspase 8 is activated in a different manner to caspase 9, usually by a variety of death receptors, including type 1 tumour necrosis factor receptor (TNF-R1) and the Fas receptor (Earnshaw et al., 1999). Caspase 8 is also known to activate caspase 3 *in vitro* (Stennicke et al., 1998) which may explain why no other downstream caspases have yet been detected in psychosine-mediated apoptosis. The presence of both caspase 8 and 9 indicates that more than one apoptotic pathway may be mediated by psychosine.

5.1.5. Secretory phospholipase A$_2$

Phospholipases A$_2$ (PLA$_2$s) are a subgroup of phospholipases, enzymes which play an important role in phospholipid metabolism. PLA$_2$s catalyse the hydrolysis of phospholipids to produce arachidonic acid (AA) and lysophosholipids. There are three broad families of PLA$_2$ enzymes, secretory PLA$_2$s (sPLA$_2$s), cytosolic PLA$_2$s and Ca^{2+} independent PLA$_2$s (Murakami and Kudo, 2002). Despite the

name, sPLA$_2$s can act both intracellular and extracellularly (through receptors) and their expression is known to be stimulated during inflammation. The expression of sPLA$_2$s can be induced by proinflammatory cytokines, such as TNF-α and interleukin 1-beta (Capper and Marshall, 2001).

A study by Giri et al. (2006) detected an increase in one of the products of sPLA$_2$ activity, lysophosphatidylcholine, in the brains of both Krabbe patients and *twitchers*. Also, AA, a major product of sPLA$_2$ activity, was found to be elevated in cultured oligodendrocytes incubated with psychosine (Giri et al., 2006). Inhibition of sPLA$_2$ activity in psychosine treated cells resulted in a significant reduction in AA release and a complete block of lysophosphatidylcholine production, indicating that psychosine mediates the activation of sPLA$_2$. Metabolism of AA in an inflammatory environment can produce proinflammatory prostaglandins and also reactive oxygen species (ROS) which cause oxidative stress (Toborek et al., 1999). Oxidative stress can, in turn, stimulate the activation of apoptotic stress signal pathways. An increase in ROS in psychosine treated cells is in agreement with the finding by Haq et al. (2003) that levels of glutathione, a scavenger of oxygen free radicals, are reduced in psychosine treated oligodendrocytes. Indeed, inhibition of sPLA$_2$ in psychosine treated oligodendrocytes was found to attenuate activation of the stress activated JNK and p38 MAPK pathways (Giri et al., 2006), which suggests that ROS, via sPLA$_2$ activation, plays a role in their activation in the disease state.

There are several groups of sPLA$_2$ and the specific one that is activated by psychosine is not yet known. A strong candidate is sPLA$_2$-IIA, which is regarded as the inflammatory sPLA$_2$ (Fuentes et al., 2002). sPLA$_2$-IIA has been shown to enhance TNF-α induced sPLA$_2$-IIA expression via an autocrine loop which involves peroxisome proliferator-activated receptor alpha (PPAR-α) (Beck et al., 2003). PPAR-α are members of the nuclear receptor superfamily and can bind products of sPLA$_2$-IIA activity (Corton et al., 2000). Ligand activation of PPAR-α regulates the expression of peroxisomal genes, lipid metabolism and cell growth (Corton et al., 2000).

5.1.6. Peroxisomal dysfunction

Peroxisomes are subcellular organelles that perform many functions, including synthesis of plasmalogens, a major lipid component of myelin, β-oxidation of fatty acids and metabolism of ROS (Corton et al., 2000; Schrader and Fahimi, 2004). Two peroxisomal enzymes, acyl-CoA oxidase and alkyl-DHAP synthase, have been observed to decrease in *twitcher* brains over time compared with age-matched control brains (Haq et al., 2006). This concurs with Khan et al. (2005) who found that β-oxidation was reduced in *twitcher* brains and that treatment of primary oligodendrocyte cultures with psychosine reduced levels of alkyl-DHAP synthase. While Haq et al. (2006) found that the peroxisomal enzyme catalase, which detoxifies hydrogen peroxide, remained at normal levels in the *twitcher* brain, a later study by Contreras et al. (2008) found that the catalase levels in *twitcher* livers were decreased relative to controls. This may be attributable to the greater amount of myelin, and thus psychosine and inflammatory stimuli, in the *twitcher* PNS relative to the CNS.

PPAR-α regulates the transcription of peroxisomal enzymes and this receptor was also found to be decreased in *twitcher* brains (Haq et al., 2006). This is somewhat contradictory to the fact that sPLA$_2$-IIA has been reported to exert its effect through PPAR-α activation (Farooqui et al., 2007). That is, if the sPLA$_2$ activated by psychosine is sPLA$_2$-IIA, then there should be increased levels of PPAR-α rather than decreased levels. Inhibition of sPLA$_2$ reduced the degree of down-regulation of PPAR-α in psychosine treated oligodendrocytes (Haq et al., 2006) indicating that dysfunction of peroxisomal functions in the *twitcher* may be due to psychosine-mediated activation of sPLA$_2$. Given the important role of many peroxisomal enzymes in ROS synthesis and degradation (Schrader and Fahimi, 2004), dysfunction of these organelles could lead to alterations in the expression of these enzymes to favour either a net increase or decrease in ROS. The former outcome is supported by depleted levels of glutathione in both psychosine treated oligodendrocytes and in the *twitcher* brain (Haq et al., 2003; Khan et al., 2005).

5.1.7. Prostaglandin D$_2$

Prostaglandin D$_2$ (PGD$_2$) is a metabolite of AA that plays a role in mediating inflammatory responses (Kanaoka and Urade, 2003). Oxidation of AA by cyclo-oxygenase enzymes produces PGH$_2$ which decomposes to PGD$_2$ and PGE$_2$ (Giles and Leff, 1988). Production of PGD$_2$ from PGH$_2$ is enhanced by the enzymatic action of hematopoietic PGD synthase (HPGDS) (Giles and Leff, 1988). In the *twitcher*,

activated microglial expression of HPGDS is upregulated with disease progression, and cell surface receptors for PGD_2, DP_1 and DP_2, are upregulated in activated astrocytes (Mohri et al., 2006). Inhibition of signalling through the HPGDS/ PGD_2/DP_1 pathway reduces the severity of astrocytosis and demyelination in the *twitcher* and as such, this pathway has been proposed to enhance these pathological signs in Krabbe disease (Mohri et al., 2006).

5.2. Immune system involvement

5.2.1. Inflammatory responses

Myelin and/or oligodendrocyte debris produced by oligodendrocyte death in Krabbe disease activates microglial cells, resident macrophages in the brain, which are the primary mediators of neuroinflammation (Farooqui et al., 2007). Activated microglia become phagocytic and secrete a variety of cytokines, including the proinflammatory cytokines, TNF-α, interleukin 1-beta and interferon-gamma. These cytokines augment and perpetuate microgliosis (Merrill and Benveniste, 1996) as well as induce astrocytes, another glial cell, to become hypertrophic and undergo reactive astrocytosis (Back and Volpe, 1998). The chemokines, macrophage chemotactic protein-1 and macrophage inflammatory protein-1 alpha, secreted from activated microglia and astrocytes act in concert with cell adhesion molecules to recruit blood-borne macrophages to the site of injury in the CNS (Wu et al., 2001; Wu and Proia, 2004), which further contribute to the inflammatory milieu in the nervous system. The extent of microgliosis, astrocytosis and macrophage infiltration increases in the *twitcher* CNS as demyelination progresses (Suzuki, 2003b). However, the rate of microglial/macrophage proliferation declines in the later stages of the disease (Ohno et al., 1993b). The blood-brain barrier appears to remain intact in *twitchers* (Kondo et al., 1987). This is possibly due to the suppressive effect interleukin-6 (produced by microglia/macrophages and astrocytes) has on TNF-α production (Schindler et al., 1990), which is known to cause damage to the blood-brain barrier (Merrill and Benveniste, 1996). Infiltrating T lymphocytes are negligible in the *twitcher* brain (Suzuki and Taniike, 1995) unlike other neuroinflammatory diseases such as multiple sclerosis (MS), although microglia/macrophages expressing MHC class II (Ia) molecules are present in the *twitcher* CNS and PNS (Ohno et al., 1993a). Engineered Ia deficient *twitchers* had reduced neuropathology and milder clinical signs (Matsushima et al., 1994). While the classical function of Ia molecules is to present antigens to CD4^{+} (helper) T cells, there is a second function of Ia molecules, which is to promote cell proliferation and differentiation through activation of transmembrane signalling pathways (Matsushima et al., 1994). This function is in agreement with the observed pathology in Ia deficient *twitchers*.

5.2.2. Tumour necrosis factor-alpha

TNF-α plays a role in both apoptotic and anti-apoptotic signalling pathways (Natoli et al., 1998). This cytokine can act through two cell surface receptors, although the majority of known TNF-α biological functions, including the induction of cytokine production and apoptosis, are mediated through TNF receptor 1 (TNF-R1) (Natoli et al., 1998; Pedchenko et al., 2000). Binding of TNF-α to TNF-R1 can induce a cytoprotective effect by signalling downstream molecules, which results in translocation of the transcription factor NF-κB to the nucleus where it activates transcription of genes that suppress apoptosis (Natoli et al., 1998). An alternative signalling pathway can be activated upon binding of TNF-α to TNF-R1, which leads to cleavage of apoptotic proteases (Natoli et al., 1998). It is possible that the down-regulation by psychosine of NF-κB transactivation (Haq et al., 2003) in Krabbe disease unbalances these two pathways, leading to a relative increase in apoptotic signalling by TNF-α and an increase in oligodendrocyte death.

The expression of TNF-α is significantly increased in the CNS of *twitcher* mice (LeVine and Brown, 1997) and Krabbe patients (Pasqui et al., 2007). TNF-α can induce apoptosis of oligodendrocytes *in vitro* (Akassoglou et al., 1998) and is suspected of playing a pathogenic role in Krabbe disease by inducing expression of Ia molecules (Matsushima et al., 1994). TNF-α inhibition in experimental auto-immune encephalomyelitis (EAE), an animal model of MS, reduces clinical symptoms (Baker et al., 1994). However, *twitcher* mice deficient for TNF-R1 do not show reduced clinical or pathological signs, which suggests that TNF-α does not play a major role in the pathophysiology of this disease (Pedchenko et al., 2000). In contrast, a study by Kagitani-Shimono et al. (2005) found that TNF-R1 significantly increased in *twitcher* brains compared with normal mice and that inhibition of TNF-α

reduced the severity of demyelination in *twitchers*. Further research is required to elucidate the exact role played by TNF-α in Krabbe disease.

5.2.3. Inducible nitric oxide synthase

Reactive nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS), the induction of which can be increased under inflammatory conditions (Farooqui et al., 2007). The expression of iNOS in peroxisomes can be induced by TNF-α and interferon-gamma (Stolz et al., 2002). In addition, the appearance of iNOS in peroxisomes has been associated with reduced catalase activity (Stolz et al., 2002), an enzyme responsible for degrading hydrogen peroxide generated by the oxidative reactions that occur in peroxisomes (Schrader and Fahimi, 2004). Elevated concentrations of NO can cause nitrative stress, overwhelming the anti-oxidative defences of oligodendrocytes (Smith et al., 1999) and resulting in necrotic death (Mitrovic et al., 1995). iNOS is not expressed in the normal brain but it has been detected in the brain of human Krabbe patients (Giri et al., 2002) as well in patients with other neuroinflammatory diseases, including MS (Bagasra et al., 1995) and Alzheimer's disease (Vodovotz et al., 1996). Cytokine induced NO production by microglial cells has been proposed as a mechanism of oligodendrocyte death in multiple sclerosis (Merrill et al., 1993). Astrocytes, microglia and macrophages can all express iNOS in response to proinflammatory cytokines (Marks-Konczalik et al., 1998).

Psychosine has been shown to potentiate cytokine-mediated iNOS induction in C6 glial cells in a dose dependent manner (Giri et al., 2002). Explanations for this potentiation have also been proposed; psychosine alone can modulate the nuclear translocation of activator protein (AP) -1 (Giri et al., 2002), a transcription factor which plays a role in regulating cytokine-mediated iNOS induction (Marks-Konczalik et al., 1998). Psychosine also has an additive effect on cytokine induction of C/EBP-δ (Giri et al., 2002), another transcription factor which is induced by interleukin 1-beta and promotes expression of iNOS (Kolyada and Madias, 2001).

6. Treatment

6.1. Cell replacement strategies

There are no curative treatments for Krabbe disease in humans. Bone marrow transplantation (BMT) is currently the most effective way of treating Krabbe disease. As the pathology of the disease is largely confined to the brain, it relies on hematopoietic stem cells from the donor marrow differentiating into monocytes and crossing the blood-brain barrier to contribute to the turnover of the microglial population (Krivit et al., 1995b). Donor-derived microglia with functional enzymatic activity can then begin to clear accumulated substrate. Enzyme transfer from microglia to surrounding cells also assists in this process (Vellodi, 2005). While the treatment of late-onset Krabbe disease with BMT can result in clinical remission, treatment of the more common infantile disease has been less successful (Krivit et al., 1995a; Krivit et al., 1998). The difference in success can be explained by the rapid disease progression in the latter case combined with slow microglial engraftment (Vellodi, 2005). The best responses of infantile Krabbe disease to BMT have been achieved when transplantation occurs prior to the onset of clinical signs (Krivit et al., 1999). As the introduction of prenatal or newborn screening for Krabbe disease is unlikely given the rarity of the disease, alternative treatments that are effective when administered to symptomatic patients are needed.

Neural stem cell transplantation is a potential complimentary therapy to BMT as these cells could differentiate into functional oligodendrocytes and replace those undergoing apoptosis in the brain of patients with Krabbe disease. Neural stem cell transplantation in *twitcher* mice has shown that transplanted cells can engraft, migrate and differentiate into astrocytes and oligodendrocytes (Taylor et al., 2006; Zhao et al., 2007). However, the percentage of injected cells that engrafted was very low in both control and *twitcher* brains, suggesting that additional factors which promote the migration, proliferation and survival of neural stem cells and oligodendrocyte progenitor cells may be needed to increase the effectiveness of this treatment in the *twitcher* (Zhao et al., 2007).

6.2. Gene therapy

As LSDs are caused by single gene defects they are attractive diseases for gene therapy. Advantages of gene therapy over BMT are that it could eliminate the problems associated with graft rejection and graft-versus-host disease and also eliminate reliance on donors (Sands, 2004). *In vivo* gene therapy in *twitchers* has been met with partial success but only when the vector is administered directly into the brain immediately after birth (Rafi et al., 2005; Shen et al., 2001). The use of *ex vivo* gene therapy holds promise as the injection of neural stem cells transduced to over-express galactosylceramidase into postnatal 10 day old *twitcher* brains was found to prolong survival of *twitchers* (Pellegatta et al., 2006). However, remaining challenges facing the application of gene therapy in humans include difficulties in delivering vectors to the brain via a systemic route and inadequate distribution of vectors or genetically engineered cells throughout the brain (Sands, 2004).

6.3. Growth and survival factors of oligodendrocytes

Factors which promote the growth and survival of oligodendrocyte progenitor cells and oligodendrocytes may serve as targets for the development of novel adjunctive therapies for Krabbe and other demyelinating diseases. The growth factor, leukaemia inhibitory factor promotes maturation and survival of oligodendrocytes *in vitro* (Barres et al., 1993) and endogenous leukaemia inhibitory factor has been found to reduce demyelination and oligodendrocyte death in murine EAE (Butzkueven et al., 2006). Both neurotrophin 3 and insulin-like growth factor-I have also been found to be important for oligodendrocyte growth and survival *in vivo* (Barres et al., 1994; Barres et al., 1993; Carson et al., 1993). Through microarray analysis, growth-arrest specific protein 6 has been identified as a survival factor for oligodendrocytes (Shankar et al., 2003). Subsequent studies have shown that growth-arrest specific protein 6 protects against TNF-α induced apoptosis in cultured human oligodendrocytes (Shankar et al., 2006) and also against cuprizone-induced demyelination in a murine model (Binder et al., 2008).

As neuroinflammation may contribute to apoptosis of oligodendrocytes in Krabbe disease, therapies aimed at inhibiting this inflammation may delay disease progression, allowing more time for other treatments to be administered and take effect. Treatment of *twitcher* mice with the anti-inflammatory drug ibubilast in later diseases stages reduced the severity of demyelination and clinical signs (Kagitani-Shimono et al., 2005). Inhibition of signalling via the inflammatory mediator PGD_2 also suppressed oligodendrocyte apoptosis and demyelination in the *twitcher* (Mohri et al., 2006).

Several chemokines contribute to the migration, proliferation and differentiation of neural stem cells and oligodendrocyte precursor cells. Chemokines strongly implicated in these functions include CXCL1 and CXCL12 and their respective receptors, CXCR1 and CXCR4 (Ambrosini and Aloisi, 2004; Dziembowska et al., 2005). As the efficacy of cell replacement therapies for Krabbe disease, including BMT and neural stem cell transplantation, as well as *ex vivo* gene therapy will be conditioned by the distribution and concentration of these chemokines it would be of interest to look at their expression in the disease state and whether or not this is up-regulated in an effort to promote re-myelination.

Conclusion

This review has summarised current understanding of some of the possible pathophysiological mechanisms of psychosine-mediated oligodendrocyte death in Krabbe disease. The emerging picture is that the accumulation of psychosine disrupts downstream biochemical pathways within the cell which ultimately result in cell death. Oligodendrocyte dysfunction, failure to repair myelin adequately and cell death activates a neuroinflammatory response which recruits macrophages and activates microglia, amplifying the production of cytotoxic molecules which contribute further to oligodendrocyte death. Elucidating the exact pathways that are disrupted by psychosine and the sequence of events that follow on from this disruption will expose novel targets for medical intervention to protect against the rapid depletion of oligodendrocytes in this disease. Delaying disease progression in this way may increase the effectiveness of both current and future enzyme-based therapies which aim to reduce the primary cellular storage. In addition to treatment advances, further research into the pathophysiology of Krabbe disease will potentially unveil new insights into normal cell physiology.

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