

# Krabbe Disease in the Australian Working Kelpie

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Krabbe disease is a lysosomal storage disorder that affects several species including humans, mice and dogs. It is caused by mutations in the GALC gene that encodes galactosylceramidase. These mutations result in lowered activity of this lysosomal enzyme which has an important role in myelin turnover. As a consequence, individuals with Krabbe disease show signs of neurological disease and pathological features occur in the central and peripheral nervous systems. These pathological features are globoid cells, demyelination and inflammation. Mechanisms behind the development of these features remain unclear despite genetic, biochemical, cellular and clinical studies. Genetic studies in Krabbe disease have allowed the development of PCR-based tests which have been proven to be a useful tool in identifying carriers of the disease. The recent diagnosis of Krabbe disease in the Australian Working Kelpie provides a new opportunity for genetic and pathological studies to further characterise Krabbe disease.

**Keywords:** Krabbe disease, globoid cell leukodystrophy, canine, pathology, molecular basis, PCR-based test

**Abbreviations:** CA, cerebellar atrophy; CDV, canine distemper virus; CNS, central nervous system; EMAI, Elizabeth Macarthur Agricultural Institute; FT-IR, Fourier-transform infrared microscopy; IL-6, interleukin-6; LSD, lysosomal storage disorder; MS, multiple sclerosis; OMIA, Online Mendelian Inheritance in Animals; OMIM, Online Inheritance in Man; PCR, polymerase chain reaction; PLA2, phospholipidase A2 system; PNS, peripheral nervous system; TNF- $\alpha$ , tumour necrosis factor alpha; WHW, West Highland White terrier

## Introduction

Lysosomal Storage Disorders (LSDs) are a group of severe and rapidly progressive inherited disorders that affect humans and animals (Platt and Walkley, 2004). Krabbe disease is a LSD in which the pathology and molecular basis has been investigated in humans, mice and dogs. Despite these investigations gaps in knowledge still remain, particularly in our understanding of Krabbe disease mechanisms (Suzuki, 2003; Wenger et al., 2001). The recent diagnosis of Krabbe disease in the Australian Working Kelpie provides a new opportunity to further characterise the disease (Taylor, unpublished data).

The following paper will review current knowledge of the pathology and molecular basis of Krabbe disease in humans, mice and dogs. It will also discuss investigations of the pathophysiology of Krabbe disease and the benefits of PCR-based tests for identification of carriers in both humans and animals.

## 1. Lysosomal storage disorders

Lysosomes are organelles that contain hydrolytic enzymes. These enzymes catabolise both endogenous and cell ingested material (Frandsen et al., 2003). As such, lysosomes have an important role in cell metabolism and consequently cell function. LSDs are a group of inherited diseases occurring in humans and animals when there is lowered activity of a lysosomal enzyme (Platt and Walkley, 2004). The result of this enzyme deficiency is the accumulation or storage of non-catabolised substrates. This storage initiates a cascade of pathological dysfunction typically observed in the nervous system (Platt and Walkley, 2004).

The majority of LSDs are of autosomal recessive inheritance and are rare in occurrence. The incidence of LSDs as a group in humans is 1 in 8000 live births (Meikle et al., 1999; Platt and Walkley, 2004). Despite the rarity of these diseases, they have devastating effects, and impose a significant burden on families and the community. Due to their involvement in the nervous system many LSDs result in

neurological dysfunction and mental retardation (Platt and Walkley, 2004). The treatment options for LSDs remain limited despite ongoing research (Kolter and Sandhoff, 2006; Platt and Walkley, 2004; Wenger et al., 2001).

LSDs may be classified on the basis of the substrate being accumulated or the molecular defect which causes the disease (Platt and Walkley, 2004). Table 1 summarises classification based on the accumulated substrate.

**Table 1: Categories of LSDs based on accumulated substrate**  
(Modified from Hopwood et al., 2004)

Classification	Accumulated Substrate	Disease Examples	Species
<b>Lipidoses</b>	Lipids. Includes sphingolipids, cholesterol esters and triglycerides	Krabbe disease Gaucher disease Wolman disease	Humans, mice, dogs, sheep, monkey Humans, mice, dogs, sheep Humans, mice
<b>Mucopolysaccharidoses (MPS)</b>	Mucopolysaccharides (glycoaminoglycans)	MPS I MPS II MPS III	Humans, mice, cats, dogs Humans, mice, dogs, Humans, dogs, cattle
<b>Glycogenosis</b>	Glycogen	Pompe disease	Humans, mice, cattle, dogs, cats
<b>Glycoproteinoses</b>	Glycoproteins and/or oligosaccharides	Fucosidosis $\alpha$ -mannosidoses Sialidosis	Humans, dogs Humans, cattle, mice, guinea pig, cats Humans, mice

## 2. Krabbe disease

Krabbe disease (OMIM #245200, OMIA #1140/000578), also known as globoid cell leukodystrophy, is a LSD with autosomal recessive inheritance that affects several species including humans, rhesus macaques, dogs, mice (Suzuki and Suzuki, 1985; Suzuki, 2003) and sheep (Pritchard et al., 1980). It is caused by mutations in the GALC gene that encodes the lysosomal enzyme galactosylceramidase (galactocerebroside  $\beta$ -galactosidase) (Luzi et al., 1997; Rafi et al., 1995; Sakai et al., 1996; Victoria et al., 1996). As a result of these mutations the activity of galactosylceramidase is lowered (Wenger et al., 2001) and the degradation of galactosylceramide during myelin turnover is impaired (Suzuki, 2003; Wenger et al., 2001).

Galactosylceramidase activity plays a crucial role in the catabolism of the myelin sheath of axons during myelin turnover (Wenger et al., 2001). The myelin sheath acts as an electrical insulator that increases the ability of responses to be transmitted through the nervous system (Behan, 2004). It is composed of 30% protein and 70% lipid. Of this lipid component, 29% is galactolipid, and the majority of this is galactosylceramide (galactocerebroside) (Lefebvre and Vartanian, 2002). Myelin turnover is the process by which these myelin components are broken down and replaced (Lefebvre and Vartanian, 2002). In humans this process is initiated within eighteen months of birth (Wenger et al., 2001). Myelination and myelin turnover is also initiated early in canine development occurring within two to three months of birth in domestic dogs (Lord and Duncan, 1987). The role of galactosylceramidase in myelin turnover is to catabolise the myelin lipid, galactosylceramide into galactose and ceramide (Lefebvre and Vartanian, 2002; Wenger et al., 2001). In the normal nervous system these substances are processed by the lysosome (Kolter and Sandhoff, 2006) and recycled components are able to enter the remyelination pathway (Lefebvre and Vartanian, 2002; Suzuki, 2003). Remyelination does not occur effectively in Krabbe disease (Suzuki, 2003; Wenger et al., 2001), but myelin turnover continues (Suzuki, 2003).

Galactosylceramidase also has a role in the catabolism of psychosine which is produced during myelin turnover (Suzuki, 2003). Psychosine is a cytotoxic intermediate in the biosynthesis of ceramide (Suzuki, 2003). The role of galactosylceramidase in myelin turnover means that its deficiency results in demyelination which alters neuronal conduction and so produces signs of neurological dysfunction. The course of this neurological dysfunction is rapidly progressive and fatal (Suzuki, 2003; Wenger et al., 2001). Bone marrow transplantation is one of the few treatments available to manage Krabbe disease (Suzuki, 2003; Wenger et al., 2001). However, the success of this treatment option is dependent on rapid diagnosis, it entails substantial risks and it does not cure the disease. Bone marrow transplantation only succeeds in slowing the progression of Krabbe disease and improving clinical signs (Suzuki, 2003).

## 2.1 Krabbe disease in humans

Krabbe disease in humans is typically a neurodegenerative disease of infancy, but there have been rare instances where Krabbe disease has been diagnosed in older children and even adults (Wenger et al., 2001). Symptoms of Krabbe disease usually indicate involvement of the central nervous system (CNS), particularly the cerebellum (Jacob et al., 1973; Suzuki, 2003) which controls the refinement and execution of movement and the co-ordination of muscle activity (Starr and Taggart, 2001). The disease affects all white matter tracts in the CNS and the Schwann cells of the peripheral nerves. As the disease progresses clinical signs of loss of higher cerebral functions develop (Suzuki, 2003; Wenger et al., 2001). The cerebrum is the region of the brain responsible for the initiation of movement and conscious sensation (Starr and Taggart, 2001). The usual age of onset for infantile Krabbe disease is 3-6 months and initially children show signs of hyperirritability, are hypersensitive to the external environment, and show stiffness of the limbs (Suzuki and Suzuki, 1985; Suzuki, 2003; Wenger et al., 2001). The symptoms of Krabbe disease progress rapidly as psychomotor functions deteriorate until the children become unresponsive and unaware of their surroundings. Children affected with Krabbe disease rarely survive beyond two years of age (Suzuki, 2003; Wenger et al., 2001).

Histopathological examination of the CNS in individuals affected with Krabbe disease reveals myelin degeneration, oligodendrocyte death, astrogliosis, and globoid cell formation. All pathological features are observed in the white matter of the brain, spinal cord and Schwann cells of peripheral nerves (Jacob et al., 1973; Krabbe, 1916; Schochet et al., 1976). Globoid cell formation is a particular characteristic of the disease, and when this is observed diagnosis is rarely in doubt (Yunis and Lee, 1976).

The diagnosis of Krabbe disease is currently made by a combination of methods including histopathologic examination, enzymic assay and genetic testing (Suzuki, 2003; Wenger et al., 2001). Historically the disease was diagnosed *post mortem* by histopathologic observations of central nervous tissue (Jacob et al., 1973; Krabbe, 1916; Schochet et al., 1976). This method continues to be used in conjunction with an enzyme assay to confirm diagnosis (Suzuki, 2003; Wenger et al., 2001). This enzyme assay was developed in the 1970s (Miyatake and Suzuki, 1972a; Suzuki and Suzuki, 1970) and measures the activity level of galactosylceramidase (Miyatake and Suzuki, 1972a). The activity of galactosylceramidase is used to determine the phenotype of an individual as affected or normal. While the enzyme assay is effective in diagnosing Krabbe disease, it cannot be used to conclusively identify carriers (Wenger et al., 2001). Recently, the advent of molecular technology has allowed for mutations causing the deficiency of galactosylceramidase to be identified (Fu et al., 1999; Rafi et al., 1995). The identification of these mutations has also facilitated the confirmation of Krabbe disease diagnosis through the development of PCR-based tests designed to check for the presence these mutations (Fu et al., 1999; Rafi et al., 1995; Wenger et al., 2001). Genetic testing is a particularly useful diagnostic tool in families where parents are known carriers. In this situation PCR-based tests are employed in prenatal diagnosis using a sample of amniotic or chorionic villus cells (Wenger et al., 1997).

## 2.2 Animal models

Animal models are valuable resources where investigation in human subjects may be limited by ethical concerns or limited availability. Since Krabbe disease primarily affects children ethical concerns of disease investigation in humans can be manifold (Platt and Walkley, 2004; Suzuki and Suzuki, 1985). There is also the rarity and variability of the disease which challenges experimental design and analysis (Wenger, 2000). Animal models can overcome both these difficulties, providing test subjects which can be used in studies to increase understanding and knowledge of the disease, as well as in treatment trials to improve the quality of life for affected individuals (Wenger, 2000).

Krabbe disease research has several different animal models available for investigation. These animal models include the rhesus monkey (Baskin et al., 1998; Borda et al., 2008), the sheep (Pritchard et al., 1980), the mouse (Duchen et al., 1980) and the dog (McGraw and Carmichael, 2006; Wenger et al., 1999). Of these models, the mouse model, known as the *twitcher* mouse has been studied in greatest depth (Duchen et al., 1980; Kobayashi et al., 1980; Kobayashi et al., 1982; LeVine et al., 1994; Nagara et al., 1982; Sakai et al., 1996). Despite this, it is important to consider that each animal model has their own strengths and weaknesses (Wenger, 2000), and information drawn from experiments using a single animal model can be used in conjunction with information from experiments in a different model to further the understanding of Krabbe disease.

### 2.3 The *twitcher* mouse

The spontaneous murine model of Krabbe disease, the *twitcher* mouse, was reported in 1980 by Duchen and others. The original investigation into the *twitcher* mouse confirmed that the disorder was transmitted in an autosomal recessive manner and that it was caused by a deficiency of galactosylceramidase (Duchen et al., 1980). This study also recognised the similarities between Krabbe disease in the *twitcher* mouse and humans. As a consequence, it established the *twitcher* mouse as an important animal model for Krabbe disease.

In the mouse, clinical signs of Krabbe disease begin at approximately 20 days and include weight loss, head tremors and weakness in the hind limbs (Duchen et al., 1980). These signs, particularly limb weakness, are indicative of peripheral nervous system (PNS) and spinal cord involvement. The tremors and poor coordination are common indicators of cerebellar disease that are present in all the animal models, including canine, human and murine forms of Krabbe disease. There is limited knowledge on the involvement of the PNS in human Krabbe disease, although it is a well established feature of murine and canine forms of the disease. Death in *twitcher* mice usually occurs by day 40 (Nagara et al., 1982) and no *twitcher* mouse has lived beyond three months without treatment (Duchen et al., 1980).

The histopathological features observed in the CNS of the *twitcher* mouse closely resemble those occurring in the human disease (Duchen et al., 1980; Kobayashi et al., 1980; Suzuki and Taniike, 1995). The white matter of the CNS shows demyelination, oligodendrocyte death, astrocytosis, microgliosis and globoid cell formation (Duchen et al., 1980).

The *twitcher* mouse makes a good animal model for the study of Krabbe disease since it is a naturally occurring enzymatically authentic model of the disease (Duchen et al., 1980). The small generation interval of mice makes them particularly suitable for experiments that require large numbers of affected or carrier animals (Kobayashi et al., 1980; Suzuki and Suzuki, 1985). The identification of the mutation causing Krabbe disease in the *twitcher* mouse by Sakai and others (1996) has led to the development of a PCR-based test. The test involves extracting a sample of DNA from the mice and using specific oligonucleotide primers in a PCR to amplify a region of DNA sequence that includes the disease mutation site (Sakai et al., 1996). The resultant fragment is then digested with a restriction enzyme which cleaves the DNA at the site of the mutation, before undergoing gel electrophoresis to determine the size and pattern of the resultant DNA bands. This process allows the individual mouse from which the DNA was sampled to be genotyped as a homozygous affected, heterozygous carrier or homozygous normal based on the pattern of the enzyme digestion (Sakai et al., 1996). The genotyping of mice using this test facilitates the diagnosis of Krabbe individuals prior to the onset of clinical signs and performance of experimental procedures (Biswas et al., 2002; Giri et al., 2006). The use and development of this PCR-based test is also an advantage for management of the *twitcher* model.

### 3. Krabbe disease in canines

Canine Krabbe disease was first diagnosed in West Highland White (WHW) and Cairn Terriers (Fankhauser et al., 1963; Wenger et al., 1999). It has since been reported in other dog breeds including the Irish Setter (McGraw and Carmichael, 2006), and the Bluetick Hound (Boysen et al., 1974). The reporting of Krabbe disease in these breeds has led to positive outcomes which are described in Table 2. In particular the development of a PCR-based test in the WHW and Cairn Terriers has led to the establishment of a research colony and widespread testing for carriers of Krabbe disease in this breed (Wenger et al., 1999).

The clinical signs of Krabbe disease in canines appear at approximately three months of age (Jortner and Jonas, 1968) and are similar to those of the *twitcher* mouse. Signs include muscle weakness, ataxia and paraplegia of the hind limbs (Boysen et al., 1974; Jortner and Jonas, 1968). As in the human and murine forms of Krabbe disease, the canine disease shows signs typical of a multi-focal neurological disease affecting the CNS and PNS, particularly spinal cord and cerebellum. Euthanasia is usually requested by the owners before the disease can progress to the final stages due to the severity of the disease (Jortner and Jonas, 1968; Wenger et al., 1999). In the clinical investigation on the Bluetick Hound conducted by Boysen and others (1974), the disease was allowed to progress for two months until one animal could no longer walk, being paralysed in both the hind and fore limbs. Soon after reaching this stage the animal died.

**Table 2: Benefits of Reporting Krabbe Disease in Dog Breeds**

Breeds	Benefits	Outcomes
West Highland White Terriers*	Veterinarian and breed society awareness (McGraw and Carmichael, 2006; Wenger et al., 1999; Boysen et al., 1974)	Krabbe disease does not remain undiagnosed (Jortner and Jonas, 1968; McGraw and Carmichael, 2006)
Cairn Terriers*	Identification of mutation causing Krabbe disease in the GALC gene (McGraw and Carmichael et al., 2006; Victoria et al., 1996; Wenger et al., 1999)	Development of PCR-based tests (McGraw and Carmichael, 2006; Victoria et al., 1996)
Irish Setter <sup>+</sup>	Development of PCR-based tests (McGraw and Carmichael 2006)	Carriers are identified and removed from breeding stock (McGraw and Carmichael, 2006; Wenger et al., 1999)
Bluetick <sup>#</sup> Hound		Research colonies are established (Wenger et al., 1999)

\*Research colonies established and breed screening program in place (Wenger et al., 1999). <sup>+</sup>PCR-based test available and a breed screening program for Krabbe disease is being developed (McGraw and Carmichael, 2006). <sup>#</sup> No further studies of Krabbe disease beyond the neuropathological investigation conducted by Boysen and others (1974).

The histopathological features of the canine disease are similar to those in humans, with extensive demyelination and oligodendrocyte and Schwann cell death, reactive astrocytosis and globoid cell formation (Jortner and Jonas, 1968). It has been noted in the dog that globoid cells are more prevalent in areas of severe myelin degeneration (Jortner and Jonas, 1968; Yunis and Lee, 1976). The appearance of axonal spheroids has also been noted in the histopathology of the canine disease, but not in the human disease. In the neuropathological study conducted by Jortner and Jonas (1968) they reported the irregular appearance of axons which were swollen and pale. This description matches that of axonal spheroids which have been noted in other LSDs (Crawley and Walkley, 2007; Platt and Walkley, 2004) but not in human Krabbe disease (Jacob et al., 1973; Schochet et al., 1976; Wenger et al., 2001; Suzuki, 2003).

Canine Krabbe disease may be diagnosed based on the result of the galactosylceramidase assay (Suzuki et al., 1974) or a PCR-based test where one is available for the known mutation in a breed (Wenger et al., 1999). When diagnosis is based on the enzyme assay, affected individuals have a lower galactosylceramidase activity than normal and heterozygotes (Suzuki et al., 1974). The galactosylceramidase assay is the same method used to diagnose Krabbe disease in humans (Wenger et al., 2001), and is a reliable and effective technique of identifying diseased but not carrier individuals. This technique was used by Suzuki and others (1974) to confirm that canine Krabbe disease is an enzymatically authentic model of the disease. In breeds such as the WHW Terrier, Cairn Terrier and Irish Setter where a PCR-based test is available, diagnosis may be based on this (McGraw and Carmichael, 2006; Wenger et al., 1999).

### 3.1 The canine model

The occurrence of Krabbe disease in dogs has led to their use as an animal model (Wenger et al., 1999). The canine model of Krabbe disease is more appropriate for studies involving the clinical and pathophysiological aspects of the disease (Wenger et al., 1999; Wenger, 2000). It is more suitable for these types of studies than the *twitcher* mouse because the greater size and complexity of the canine brain

allows clearer dissection of the different regions of the nervous system (Suzuki and Suzuki, 1985), correlation with clinical signs of dysfunction and more reliable comparison to the human brain (Yunis and Lee, 1976). The canine model is also useful since the behavioural changes that occur in larger animals affected with LSDs are more similar to the psychomotor changes that occur in humans (Platt and Walkley, 2004) and can be more easily monitored in an animal such as the dog which has complex social interactions with people and its environment.

#### **4. Krabbe disease in the Australian Working Kelpie**

In 1998-1999, three related Australian Working Kelpie pups from the same property in Tamworth, NSW were presented to a veterinarian with signs of neurological disease and clinical investigations were performed. In these clinical examinations, each of the pups showed signs of disease similar to the clinical signs of Krabbe disease described in WHW and Cairn Terriers by Jortner and Jonas (1968). However, since Krabbe disease had not been previously reported in the Australian Working Kelpie, the first animal presented was treated for suspected toxoplasmosis until test results were returned negative. Following the negative toxoplasmosis results the first two pups were surrendered to Elizabeth Macarthur Agricultural Institute (EMAI) for investigation of neurological disease.

At EMAI, preliminary histopathology was performed and it was found the brain tissue displayed the globoid cell lesions characteristic of Krabbe disease (Taylor, unpublished data). This prompted investigators at EMAI and the University of Sydney to conduct a galactosylceramidase assay in the third pup. It was found that the third Australian Working Kelpie pup had low galactosylceramidase activity indicative of Krabbe disease (Taylor, unpublished data).

To date, there has been no published data characterising Krabbe disease in Australian Working Kelpies. This is a concern as it means veterinarians are not aware that Krabbe disease can occur in this breed and it may go undiagnosed. This is undesirable as Krabbe disease is an inherited disorder and widespread use of a phenotypically normal heterozygote carrier over the breed could lead to increased incidences of Krabbe disease in the Australian Working Kelpie. Further, there is another inherited neurological disorder called hereditary cerebellar atrophy (CA) which affects the Australian Working Kelpie breed (Thomas and Robertson, 1989). The clinical signs of this disorder are similar to the signs seen in Krabbe disease affected Kelpies and include ataxia, hypermetria and dysmetria of the hind limbs (Thomas and Robertson, 1989). The similarity in clinical signs of CA and Krabbe disease may mean that cases of Krabbe disease are being misdiagnosed, especially since CA is known in the veterinary community and by the breed society to be a genetic disorder which affects the Australian Working Kelpie breed.

#### **5. Pathogenesis**

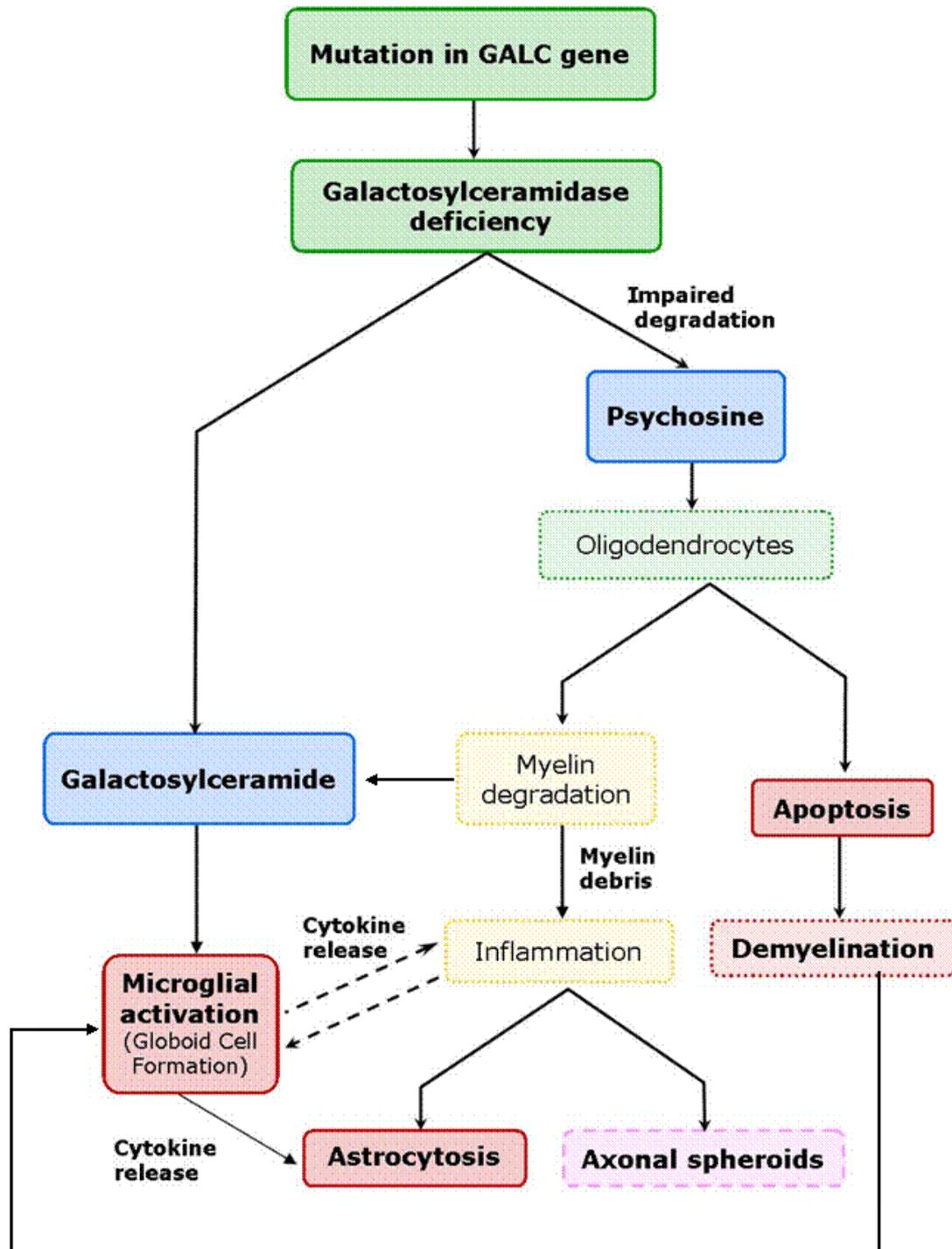
In LSDs it is important to understand how the genetic deficiency in lysosomal enzyme activity and the storage of the non-catabolised substrate are responsible for pathology (Platt and Walkley, 2004). Recently, the focus of pathological studies on LSDs has been in finding the link between the observed clinical signs of the disease and the histopathological and biochemical features (Borda et al., 2008; LeVine et al., 1994; Platt and Walkley, 2004).

The pathogenesis of Krabbe disease is caused by an underlying genetic malfunction in the production of galactosylceramidase. This leads to a deficiency of this enzyme which results in the impaired degradation of galactosylceramide, the primary substrate of galactosylceramidase, and psychosine, its secondary substrate (Suzuki, 2003). Unlike most other LSDs the primary substrate is not found at elevated levels in the nervous tissue of Krabbe affected individuals (Suzuki, 2003). This suggests that galactosylceramide is not stored and that Krabbe disease is not a typical example of a LSD. Instead, the secondary substrate has been found at high concentrations (Igisu and Suzuki, 1984; Kobayashi et al., 1980; Svennerholm et al., 1980) and is believed to cause the pathological features of the disease in what is known as the 'psychosine hypothesis' (Miyatake and Suzuki, 1972b).

##### **5.1 The pathogenic cascade**

The pathogenic cascade of Krabbe disease is outlined in Figure 1. The deficiency of galactosylceramidase results in the insufficient catabolism of both galactosylceramide and psychosine during myelin turnover (Suzuki, 2003). Galactosylceramide is a main component of myelin, which is

produced by the oligodendrocytes of the CNS and the Schwann cells of the PNS (Lefebvre and Vartnanian, 2002). During myelin turnover galactosylceramide is broken down into galactose and ceramide by galactosylceramidase (Platt and Walkley, 2004) and these products are then used in a separate remyelination pathway (Suzuki, 2003). In Krabbe disease the breakdown of galactosylceramide does not occur. Instead, galactosylceramide along with other myelin debris triggers a microglial response that results in the formation of globoid cells, a characteristic histopathological feature of Krabbe disease (Borda et al., 2008; Suzuki, 2003).



**Figure 1: Pathogenic cascade of Krabbe disease. Adapted from Suzuki (2003).** NOTE: Appearance of axonal spheroids has only been described in canine Krabbe disease.

Psychosine is also produced in the oligodendrocytes during normal myelin turnover, but is normally broken down immediately by galactosylceramidase so its cytotoxic effects are not demonstrated (Suzuki, 2003). In the Krabbe affected brain, psychosine is allowed to accumulate within the oligodendrocytes (Borda et al. 2008; LeVine, 1994; Suzuki, 2003). According to the 'psychosine hypothesis' this results in oligodendrocyte apoptosis causing demyelination, and the resulting myelin debris triggers an inflammatory response of astrocytosis and microgliosis (Suzuki, 2003).

## 5.2 Oligodendrocytes and demyelination

Oligodendrocytes are the glial cells in the CNS that produce myelin (Behan, 2004). One oligodendrocyte may have many processes that extend and wrap around a number of axons, myelinating them (Behan, 2004; Lefebvre and Vartanian, 2002). In Krabbe disease, swelling of the oligodendrocytes has been observed (Taniike et al., 1999). This swelling results in oligodendrocyte dysfunction and occurs before myelin turnover and the consequential demyelination begins (LeVine et al., 1994; Taniike et al., 1999). Dysfunctional oligodendrocytes then undergo programmed cell death known as apoptosis (Taniike et al., 1999) resulting in the reduced oligodendrocyte numbers seen in late stage disease (Duchen et al., 1980; Jortner and Jonas, 1968).

The timing of oligodendrocyte death and demyelination during brain development has been investigated in the *twitchee* mouse (LeVine et al., 1994; Taniike et al., 1999). These investigations are based on the regional difference in brain myelination rates during postnatal brain development (LeVine et al., 1994). In the study conducted by Taniike and others (1999) it was observed that oligodendrocytes that initiate myelination early in brain development were the first to undergo apoptosis. This study supports the findings of LeVine and others (1994) who reported severe oligodendrocyte death and demyelination in the brainstem and cerebellum, which are myelinated early in development, and limited evidence of oligodendrocyte death in the cerebrum until very late stage disease. The findings of LeVine and others (1994) can also be related to the clinical course of the disease in humans, mice and canines which begins with signs of PNS, spinal cord and cerebellar dysfunction (Duchen et al., 1980; Jortner and Jonas et al., 1968; Wenger et al., 2001) and progresses to show cerebral indications of disease, particularly in human Krabbe disease (Suzuki et al., 2003; Wenger et al., 2001).

It is also speculated that demyelination of axons caused by the oligodendrocyte death may result in axonal and eventual neuronal loss similar to that in longstanding lesions of multiple sclerosis (MS), a demyelinating disease of humans (Adams, 1989; Gonzalez-Scarano and Baltuch, 1999). In MS, axons that remain demyelinated eventually die back to the neuron body resulting in further neurological dysfunction. Axonal loss has not been reported in Krabbe disease (Jacob et al., 1973; Jortner and Jonas et al., 1968; Suzuki, 2003), but this is possibly due to rapid progression of the disease (Suzuki, 2003; Wenger et al., 2001) which means individuals die before axonal loss secondary to demyelination can begin.

The mechanism causing programmed cell death in the oligodendrocytes of the Krabbe affected brain is not known. It has been speculated that tumour necrosis factor alpha (TNF- $\alpha$ ), a cytokine produced by microglia may have a role in the inflammatory and immune functions (Taniike et al., 1999). However, the '*psychosine hypothesis*' is widely accepted and it is thought that the accumulation of psychosine results in oligodendrocyte death, and consequential demyelination (Formichi et al., 2007; Giri et al., 2006; Igisu and Suzuki, 1984; LeVine et al., 1994; Suzuki, 2003; Taniike et al., 1999). Both may contribute to the rapid myelin loss which occurs.

## 5.3 The psychosine hypothesis

The '*psychosine hypothesis*' was first put forward by Miyatake and Suzuki (1972b) who found that Krabbe disease patients were unable to hydrolyse psychosine. The '*psychosine hypothesis*' is supported by a large number of studies and has yet to be conclusively disproved. Igisu and Suzuki (1984), Kobayashi and others (1987) and Svennerholm (1980) have all identified increased levels of psychosine in the white matter of the Krabbe affected brain. The cytotoxic effects of psychosine have also been demonstrated in both animal models (Miyatake and Suzuki, 1972b) and *in vitro* (Giri et al., 2006).

The '*psychosine hypothesis*' is also supported by a study conducted into the pattern of psychosine accumulation using Fourier-transform infrared microspectroscopy (FT-IR). FT-IR allows the accumulation of psychosine to be measured *in situ* (LeVine et al., 1994). It was found that psychosine

accumulated most in the hindbrain, a site where myelination occurs early in development (LeVine et al., 1994). The next area which had a high concentration of psychosine was the cerebellum. The cerebrum had the lowest concentration of psychosine. This pattern of psychosine accumulation matched the pattern of oligodendrocyte apoptosis also found in this study. The pattern of psychosine accumulation found in this study correlates with both the myelination and myelin turnover pattern of the developing brain and the clinical course of Krabbe disease. Myelination and myelin turnover begins in the cervical spinal cord and proceeds rostrally through the brainstem to the cerebrum (LeVine et al., 1994; Lord and Duncan, 1987). The clinical course of Krabbe disease shows motor dysfunction due to spinal cord and PNS involvement, and ataxia and weakness indicative of cerebellar disease. In human Krabbe disease these symptoms progress to cerebral signs of disease as psychomotor development and learned behaviours regress in the terminal stages (Suzuki, 2003).

Recent research into the mechanism behind apoptosis of oligodendrocytes also supports the basic premise of the '*psychosine hypothesis*.' In a recent study it was proposed that the increased levels of psychosine in Krabbe disease results in the activation of pro-inflammatory cytokines, such as TNF- $\alpha$  and interleukin-6 (IL-6) and it is in this way that psychosine induces oligodendrocyte apoptosis (Formichi et al., 2007). This proposal was based on the level of cytokines observed in psychosine treated peripheral blood leukocytes and peripheral blood monocytes (Formichi et al., 2007). Similarly, in a separate study, psychosine was also found to be an apoptosis activator (Giri et al., 2006). However, in this investigation it was found that psychosine activates the phospholipidase A2 system (PLA2) and this mediates apoptotic oligodendrocyte death (Giri et al., 2006). This finding was based on the accumulation of lysophosphatidylcholine (LPC), a product of PLA2, in the brains of Krabbe affected mice and humans, and the dose-dependant increase of LPC in cultured cells treated with psychosine (Giri et al., 2006). It is possible that there is a link between the two studies and the activation of the PLA2 system may be due to the cytokines activated by psychosine. However, further research is required before a solid conclusion can be drawn on this hypothesised pathogenic mechanism.

#### **5.4 Microglial activation**

Microglia are the resident macrophages of the CNS (Behan, 2004). The multinucleate globoid cells of Krabbe disease are believed to be activated microglia that phagocytose free galactosylceramide and other myelin degradation products. However, there has been some debate about the origin of globoid cells due to their perivascular location (Alroy et al., 1986a; Alroy et al., 1986b; Yunis and Lee, 1976). Some investigators believe that globoid cell formation could be due to the invasion and fusion of blood-derived macrophages which cross the blood-brain barrier (Alroy et al., 1986a; Borda et al., 2008). It is thought that by understanding the origin of globoid cells their role in the inflammatory response of Krabbe disease will become clear, along with potential avenues for ameliorating the severity of the disease (Borda et al., 2008).

In a recent study by Borda and others (2008) biochemical markers were used in order to determine the origin of globoid cells. This study found that globoid cells contained markers specific to both resident microglia and blood-derived macrophages. This suggests that globoid cells consist of both types of macrophages. The possibility that both types of macrophages are involved in globoid cell formation means that cytokines released from microglia could have a role in the inflammatory response observed in Krabbe disease.

The release of cytokines such as TNF- $\alpha$  and IL-6 by microglia has been well documented in several other demyelinating diseases such as MS, experimental autoimmune encephalomyelitis and canine distemper virus (CDV) (Gonzalez-Scarano and Baltuch, 1999; Grone et al., 2000; Markus et al., 2002). Cytokines released by microglia may be pro- or anti-inflammatory, amplifying the effects of inflammation as well as protecting the CNS (Gonzalez-Scarano and Baltuch, 1999; Grone et al., 2000). It has also been speculated that microglia have a role in the normal clearance of myelin break down products and may release TNF- $\alpha$  and IL-6 in this process (Borda et al., 2008). In Krabbe disease the release of these cytokines may result in the formation of globoid cells as well as other pathological features of the disease (Borda et al., 2008).

#### **5.5 Astrocytosis**

Normal astrocytes in the brain contribute to the formation of the blood brain barrier, and maintain nutrition and ion concentrations essential to neuronal function (Behan, 2004). Reactive astrocytosis

occurs when astrocytes proliferate in response to brain injury (Behan, 2004; Little and O'Callaghan, 2001). This reactive astrocytosis is what has been observed in histopathological examinations of brain tissue from individuals affected with Krabbe disease (Jacob et al., 1973; Jortner and Jonas, 1968; Itoh et al., 2002; Yunis and Lee 1976). It is thought that reactive astrocytosis occurs in an effort to 'seal off' damaged tissues and prevent spread of the injury (Buffo et al., 2008). This may be effective and useful in an infectious disease such as CDV (Markus et al., 2002). However investigators remain unsure of the function of reactive astrocytosis in a genetic disorder like Krabbe disease, where neuropathology is global throughout the nervous system.

The mechanisms behind the initiation of reactive astrocytosis in Krabbe disease are also unclear, although this is a common finding in most lysosomal storage disorders. It is thought that astrocytosis is triggered by the release of cytokines (Itoh et al., 2002; Little and O'Callaghan, 2001). However, it is not known if these cytokines are released by the microglia (Itoh et al., 2002) or normal astrocytes which are reacting to the presence of apoptotic oligodendrocytes (Little and O'Callaghan, 2001).

## 5.6 Axonal spheroids

Axonal spheroids are swellings of axons. They occur in both the white and grey matter of the CNS and PNS (Platt and Walkley, 2004). They have been noted in many neuropathies including several LSDs (Platt and Walkley, 2004) but have only been observed in the canine form of Krabbe disease (Jortner and Jonas, 1968). As a consequence of this restricted occurrence there is limited knowledge on the formation of axonal spheroids in Krabbe disease. In the majority of neuropathies featuring spheroidosis including other lysosomal disorders, the axonal spheroids are believed to be an indicator of axonal transport dysfunction (Crawley and Walkley, 2007; Itoh et al., 2006).

## 6. Investigation of pathophysiology

To have a full understanding of a genetic disorder, the pathophysiology of the disease must be investigated at several different levels. These levels include investigation of the underlying genetic defect, biochemical studies of the proteins and enzymes involved in disease pathogenesis, cellular studies of the structures involved in the pathology and clinical examinations of the individuals.

### 6.1 Genetic studies

Genetic investigations into inherited disorders like LSDs are able to increase understanding by ascertaining what causes the underlying enzyme or protein deficiency involved. Methods which have been used in genetic studies of Krabbe disease include gene mapping (Zlotogora et al., 1990), protein purification (Chen and Wenger, 1993; Sakai et al., 1994), mutation analysis (Rafi et al., 1995; Victoria et al., 1996) and *in vitro* expression studies (Victoria et al., 1996).

Protein purification and sequencing has allowed the cDNA of the GALC gene to be cloned and sequenced facilitating mutation analysis (Chen et al., 1993; Sakai et al., 1994; Sakai et al., 1996; Victoria et al., 1996). Protein sequence was back translated into mRNA to obtain the cDNA nucleotide sequence (Sakai et al. 1994). The purification of galactosylceramidase was difficult and the first studies attempting to do this reported very low yields (Chen et al., 1993; Chen and Wenger, 1993; Sakai et al., 1996). This was the main difficulty in attempts to sequence the GALC gene which was one of the last lysosomal enzyme genes to be sequenced.

Once the GALC cDNA was cloned and sequenced, mutation analysis was conducted. The aim of mutation analysis is to identify what changes in the gene cause the enzyme deficiency. In Krabbe disease over forty human mutations have been identified (Fu et al., 1999; Wenger et al., 1997) and two canine mutations have been identified (McGraw and Carmichael, 2006; Victoria et al. 1996). Mutation analysis can be linked to biochemical and expression studies and can be used to design PCR-based tests such as those available for human mutations, the *twitcher* mouse and the WHW and Cairn terrier.

Expression studies are generally conducted *in vitro* to confirm that mutations identified do lead to enzyme deficiency and are not simply polymorphisms. An expression study confirmed that one of the transversions identified in the GALC gene of the WHW and Cairn Terriers was a disease causing mutation, while the other transversion was a polymorphism that did not affect galactosylceramidase expression (Victoria et al., 1996).

PCR-based tests are used to genotype individuals and are most useful in identifying phenotypically normal carriers of a genetic disorder such as Krabbe disease. PCR-based tests involve taking a DNA sample from a suspected carrier. The DNA sample may be derived from blood (Victoria et al., 1996), tissue (Sakai et al., 1994), saliva or amniotic cells in the case of pre-natal detection of a disorder (Wenger et al., 2001). The sample forms the basis for a PCR along with specific oligonucleotide primers which incorporate a restriction site, to amplify the region of DNA sequence that includes the disease mutation site identified by the mutation analysis (Victoria et al., 1996; Sakai et al., 1994). This produces a fragment of DNA which may then be digested with a restriction enzyme as in the PCR-based tests for the *twitcher* and WHW and Cairn terrier mutations (Victoria et al., 1996; Sakai et al., 1994). In the *twitcher* and WHW and Cairn terrier DNA tests, the restriction enzyme cuts a fragment with an affected allele, while the fragment containing the normal allele remains whole (Victoria et al., 1996; Sakai et al., 1994). These digested fragments then undergo gel electrophoresis to determine the genotypes.

## 6.2 Biochemical studies

Biochemical studies are useful in pathophysiological investigations because they can identify the enzymes, substrates and mediators involved in the disease. They can also be used to confirm the enzyme deficiency, relating back to genetic studies and can contribute to understanding in cellular investigations. Methods of biochemical investigation include enzyme assays and expression studies.

Enzyme assays such as the galactosylceramidase assay which is used to confirm Krabbe disease diagnosis can be used in biochemical studies to establish enzyme activity levels in a variety of different tissues (Miyatake and Suzuki, 1972a). This establishes where the deficiency is located and if it affects the whole body or just one area or organ system. Assays can also be used to isolate biochemicals involved in the disorder. In Krabbe disease the isolation of psychosine in the brain of Krabbe affected patients but not in controls established that psychosine was involved in the disorder (Igisu and Suzuki, 1984; Svennerholm et al., 1980).

Expression studies into the role of psychosine have established the role of substrates and the pathways they trigger (Giri et al., 2006). This increases understanding of the effects biochemical pathways have on cellular metabolic pathways.

## 6.3 Cellular investigation

Investigation into the pathophysiology of a disease using cellular investigation techniques can assist in establishing the location of biochemical lesions in LSDs. Cellular investigations can also be used to study the structure and function of normal cells (Platt and Walkley, 2004) since most cellular studies report the changes in cells due to disease (Jacob et al., 1973; Jortner and Jonas, 1968). Cellular investigations can also be used to investigate the timing of pathological changes in tissues (LeVine et al., 1994) and these might be correlated to changes observed in clinical examinations. In Krabbe disease cellular structure has been investigated using a variety of histological techniques including light microscopy, electron microscopy, and immunohistochemistry. The histological and immunohistochemical techniques which have been successfully used in the cellular investigation into the pathophysiological features of Krabbe disease are outlined in Table 3.

## 6.4 Clinical examination

Clinical examination contributes to the pathophysiological investigation and allows repeated assessment of the impact of disease on neural control of sensation and movement. Clinical examinations are most useful when used in context with the other methods of investigation.

To gain a full understanding of Krabbe disease pathophysiology investigations need to examine several different aspects of the disease. The nature and difficulties of Krabbe disease research means that a full study linking each of these methods of investigation has not been attempted. This can make it difficult to integrate studies which only focus on one of these areas.

**Table 3: Histological Techniques Commonly Used in the Investigation of Krabbe Disease**

Stain	Histological/ Histochemical/ Immunohistochemical and Technique	Use
Hematoxylin and Eosin (H+E)	Histological stain, used in conjunction with light microscopy	Identifies general tissue structure (Jortner and Jonas, 1968; Wenger et al., 1999) and can be used to highlight the appearance of globoid cells (Wenger et al., 1999; Yunis and Lee, 1976)
Periodic Acid-Schiff (PAS)	Histological stain, used in conjunction with light microscopy	Binds to glycogen stores (Yunis and Lee, 1976) Globoid cells are PAS positive (Jortner and Jonas 1968; LeVine et al., 1994; Yunis and Lee, 1976)
Luxol Fast Blue (LFB)	Histological stain, used in conjunction with light microscopy	Identifies myelin and indicates areas of demyelination (Borda et al., 2008; Yunis and Lee, 1976)
Myelin Basic Protein Antibody (MBP)	Immunohistochemical/antibody stain, used in conjunction with light microscopy	Identifies myelin (Itoh et al., 2002)
Glial Fibrillary Acidic Protein Antibody (GFAP)	Immunohistochemical/antibody stain, used in conjunction with light microscopy	Identifies astrocyte hypertrophy (LeVine et al., 1994)
Lectin Stains	Histochemical, used in conjunction with light and electron microscopy	Binds to specific carbohydrates on cellular structures and has been used to identify carbohydrates within storage bodies (Alroy et al., 1986a, 1986b) In Krabbe disease lectin staining was used in an attempt to identify the nature of globoid cell inclusions
Ubiquitin Antibody	Immunohistochemical/antibody stain used in conjunction with light microscopy	Identifies axonal spheroids in which ubiquitin is accumulated (Crawley and Walkley, 2006; Platt and Walkley, 2004)

## 7. Molecular basis of Krabbe disease

Galactosylceramidase is encoded by the GALC gene in both humans and animals (Wenger et al., 1997). It was mapped to human chromosome 14, mouse chromosome 12 (Zlotogora et al., 1990) and dog chromosome 8 (NCBI, 2008). There is significant homology in the gene sequence between the three species and there are close structural similarities between the human, mouse and dog GALC genes (Sakai et al., 1996; Victoria et al., 1996; Wenger et al., 2000).

### 7.1 Human GALC mutations

Mutation analysis of the human GALC gene was facilitated by the cloning and sequencing of GALC cDNA (Chen et al., 1993; Rafi et al., 1995; Sakai et al., 1994). This allowed DNA obtained from Krabbe affected individuals to be sequenced and analysed against the normal GALC gene. To date there have been over forty mutations identified that cause the galactosylceramidase deficiency of Krabbe disease (Fu et al., 1999; Wenger et al., 1997). The most common mutation in the European population is a 30kb deletion which is associated with a C to T transversion at cDNA position 502 (Rafi et al., 1995; Wenger et al., 1997). The C to T transversion was a polymorphism which does not affect galactosylceramidase activity. The large 30kb deletion affects the production of galactosylceramidase since it removes a significant portion of the enzyme coding region (Rafi et al., 1995). In the Japanese population, the most common mutation is a 12bp deletion with a 3bp insertion. This results in the deletion of 5 amino acids and the insertion of 2 amino acids which impacts on the quaternary structure of galactosylceramidase (Tatsumi et al., 1995; Wenger et al., 1997).

The large number of mutations means that there are also a large number of possible genotypes. This means that compound heterozygotes may occur (Wenger et al., 1997). Compound heterozygotes are individuals which have two different mutated alleles at the same locus. In many cases where compound heterozygotes are identified they have low to intermediate galactosylceramidase activities and may not present with clinical signs of Krabbe disease or they may present with clinical signs which are atypical (Wenger et al., 1997). This has made it difficult to correlate phenotype with genotype in human Krabbe disease (Fu et al., 1999; Wenger et al., 1997; Wenger et al., 2000).

This difficulty in correlating phenotype with genotype may also limit the efficacy of PCR-based tests as a definitive diagnostic tool where human Krabbe disease may be suspected in an individual with no family history of the disease (Wenger et al., 2000). Despite this, PCR-based tests remain a helpful tool in confirming diagnosis and in research situations (Wenger et al., 2000). These tests are especially useful in families that are known to carry a mutation for Krabbe disease. In this situation PCR-based tests have been used to identify the genetic status *in utero* from a sample of DNA collected from amniotic fluid (Wenger et al., 1997).

## 7.2 Canine GALC mutations

Two different mutations in the canine GALC gene have been identified in three breeds (McGraw and Carmichael, 2006; Victoria et al., 1996). It is likely, considering the genetic diversity of dog breeds and the number of mutations in the genetically diverse human population, that further mutations identified in other dog breeds will be unique.

The first canine mutation was identified in the WHW and Cairn Terriers when the canine GALC cDNA was cloned (Victoria et al., 1996). The canine GALC cDNA is similar to the human GALC cDNA with 88-90% identity (Victoria et al., 1996). The mutation causing Krabbe disease in the WHW and Cairn Terriers was an A to C transversion which resulted in hydrophobic tyrosine being substituted with hydrophilic serine (Victoria et al., 1996). This amino acid substitution caused a structural change to galactosylceramidase that resulted in the enzyme having no biological activity (Victoria et al., 1996).

The second canine mutation was identified in an Irish Setter family by cDNA mutation analysis (McGraw and Carmichael, 2006). It is different from the 'terrier' mutation since it is a 78 nucleotide insertion that results in the absence of a stop codon in the open reading frame (McGraw and Carmichael, 2006). Given the mutation, the mRNA is unlikely to be translated into functional protein, but it could result in the altered mRNA being translated into a larger than normal enzyme. Enzyme expression studies have shown that if galactosylceramidase is translated it does not have any biological activity (McGraw and Carmichael, 2006).

## 7.3 Availability and use of PCR-based tests

The identification of mutations in the GALC gene has allowed PCR-based tests to be developed (Fu et al., 1999; McGraw and Carmichael et al., 2006; Sakai et al., 1996; Victoria et al., 1996; Wenger et al., 1997). These PCR-based tests are used to confirm the genetic status of an individual using a small sample of DNA (Wenger et al., 1997).

In research situations PCR-based tests are used to ascertain the genetic status of animals before the onset of clinical signs (Biswas et al., 2002; Giri et al., 2006; Wenger et al., 2001), allowing experimental procedures to be performed in the early stages of disease progression. This is particularly important when conducting treatment trials (Suzuki, 2003; Wenger et al., 2001) since it has been found in humans that for treatments such as bone marrow transplant and enzyme replacement therapy to have some effect they must be commenced as early as possible, preferably before the onset of clinical signs (Wenger et al., 1999).

The identification of carriers using PCR-based tests is particularly useful in both human and animal situations since carriers cannot be identified reliably using the enzyme assay (Wenger et al., 1997; Suzuki et al., 1972). In humans, the identification of carriers is used in genetic counselling (Wenger et al., 2001) since the birth of a child with Krabbe disease can be a devastating and heart-wrenching event for families (Jacob et al., 1973). The identification of carriers in animals by PCR-based tests has led to an implementation of a breeding program in the WHW and Cairn Terriers (Wenger et al., 1999). In this breeding program identified carriers are removed from the companion animal breeding stock and may be

referred to researchers for the ongoing establishment of a research colony (Wenger et al., 1999). A similar process occurred in the Irish Setter breed, but no research colony has been established (McGraw and Carmichael, 2006). The removal of carrier animals from breeding stock is done with the aim of eradicating Krabbe disease from these breeds (Wenger et al., 1999).

## Conclusion

PCR-based tests are important tools in managing Krabbe disease in human and animal situations. They have been developed through the identification of mutations causing Krabbe disease in the GALC gene which encodes galactosylceramidase, the lysosomal enzyme which is deficient in this disease. These genetic studies along with biochemical and cellular investigations have led to increased understanding of the pathogenesis of Krabbe disease. However, despite this increased understanding there still remain gaps in the knowledge and many mechanisms behind events in the pathogenic cascade of Krabbe disease have not been elucidated. The occurrence of Krabbe disease in another dog breed provides an opportunity to further increase the understanding of Krabbe disease pathology. It is possible that the mutation underlying Krabbe disease in the Kelpie may be identified in the future by sequencing the GALC gene in the affected dogs. Mutation analysis and development of a PCR-based test may then lead to the implementation of a heterozygote carrier screening program similar to that in WHW and Cairn terriers enabling the control and eradication of Krabbe disease from Australian Working Kelpies.

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