EX VIVO GENE THERAPY FOR THE PRESERVATION OF RETINAL AND CENTRAL NERVOUS SYSTEM STRUCTURE AND FUNCTION IN A CANINE MODEL OF CLN2 NEURONAL CEROID LIPOFUSCINOSIS

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**EX VIVO GENE THERAPY FOR THE PRESERVATION OF RETINAL AND CENTRAL NERVOUS SYSTEM STRUCTURE AND FUNCTION IN A CANINE MODEL OF CLN2 NEURONAL CEROID LIPOFUSCINOSIS**

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ABSTRACT

The neuronal ceroid lipofuscinoses (NCLs) are an inherited group of related lysosomal storage disorders. The NCLs are characterized in general by the accumulation of autofluorescent lysosomal storage material (ceroid-type lipofuscinosis) and progressive neurodegeneration. Age of onset and disease duration differs among the various forms of NCL, as each is caused by mutations in one of at least 13 different genes. Typically, the NCLs as a whole are inherited in an autosomal recessive manner, though at least one autosomal dominant form exists. Age of onset generally occurs sometime between early to late childhood, though adult onset NCL has been seen rarely. Progressive loss of neurological function is present among all forms, with progressive vision impairment also very common. Ultimately, following symptom progression, the NCLs result in premature death, with the specific age range being form dependent. In almost all cases death has been attributed to pronounced loss of neurological functions.

The CLN2 form of NCL (previously designated as classical late-infantile NCL) results from mutations in the gene \textit{TPP1} (h11p15.5), which encodes the soluble lysosomal enzyme tripeptidyl peptidase-1 (TPP1). TPP1 is a member of the sedolisin family of serine-carboxyl peptidases and is active within the
lysosome, where it cleaves N-terminal tripeptides. A long-haired miniature Dachshund model of CLN2 was developed and is currently being maintained and studied at the University of Missouri Columbia in an effort to better understand and develop therapies for CLN2. Affected dogs are homozygous for a single base pair deletion within exon 4 of *TPP1* (c.325delC) that alters the reading frame of the predicted mRNA after codon 107 and creates a premature stop codon at position 114. Ultimately, this mutation results in no functional enzyme being produced. *TPP1*⁻/⁻ dogs develop symptom progression similar to that seen in human CLN2, with ataxia, cognitive changes coinciding with cerebral atrophy, and myoclonic seizures beginning at 7-8 months of age and progressively worsening through disease end-stage by 10-11 months at which time the dogs are euthanized. Additionally, affected dogs develop vision loss that progresses to blindness with corresponding progressive retinal degeneration, electroretinogram (ERG) deficits, and the development of focal retinal detachment lesions (retinopathy). The marked similarity in disease presentation between canine and human CLN2 as well as the overall resemblance between the canine and human nervous system and retina makes these dogs an ideal model for development and evaluation of putative therapies for eventual translation to human clinical trials.

Previous studies conducted by the lab demonstrated that *TPP1* enzyme replacement therapy delivered to the CNS via cerebrospinal fluid infusion is effective in ameliorating neurological symptoms, prolonging life, and reducing the amount of lysosomal storage material within the brain; however, this therapy did
not result in the preservation of the retina. As a result, a more directed retinal therapy is necessary to prevent retinal degeneration and preserve vision. Studies were conducted utilizing a novel autologous ex vivo gene therapy approach, in which mesenchymal stem cells (MSCs) were enriched from bone marrow aspirates, transduced with viral vectors directing the constituent overexpression of TPP1, and injected intravitreally as a means of providing sustained TPP1 to the retina. Dogs were evaluated via ERG and scanning laser ophthalmoscopy (SLO) and optical coherence tomography (OCT) imaging to gauge the effectiveness of the treatment in preserving the various retinal layers and preventing the development of focal detachment lesions. The treatment resulted in preservation of retinal structure and function that appeared dose-dependent, with ERG responses remaining normal-like longer in dogs that received more cells. A similar trend was observed with regards to the CLN2-related changes in retinal structure. Regardless of dosage, the retinas of the treated eyes did eventually begin to degenerate, but at a much slower pace versus those of the contralateral control eyes. Further studies are necessary, and are in progress, to determine whether the ex vivo gene therapy approach is capable of preserving the retina beyond normal end-stage in the canine CLN2 model and if repeated therapeutic application has a beneficial effect relative to a single pre-symptomatic injection. Preliminary studies indicate that introduction of the TPP1-expressing MSCs into the cerebrospinal fluid is safe and slows central nervous system degeneration.
CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. The Neuronal Ceroid Lipofuscinoses

1.1.1. Human NCLs

Neuronal ceroid lipofuscinosis (NCL) is an umbrella term describing a group of inherited lysosomal storage disorders with similar clinical presentations and histopathological features that primarily affect the central nervous system (CNS) and the retina. In general, the NCLs are characterized by visual impairment and blindness as a result of progressive retinal degeneration and by seizures, loss of motor function, and dementia, stemming from progressive neuronal degeneration (Mole et al., 2005). For simplicity the NCLs are often referred to as Batten disease for lay audiences, although technically the term Batten disease refers only to the juvenile form of NCL, now known as CLN3, and named in recognition of the neurologist Frederick Batten who published early clinical and pathological findings of juvenile NCL (1914) and classic late infantile NCL (1915) (Mole et al., 2011). It is now known that different forms of NCL result from mutations in at least 13 different genes (OMIM PS256730). The currently accepted nomenclature for the different forms of NCL is based on the gene in which the mutation responsible for that form of NCL occurs. This nomenclature designates the different forms of NCL as CLN1 through CLN13 (OMIM PS256730). Within each disease form the phenotype can vary depending on the specific causal mutation within the gene associated with that form. For example,
a mutation that results in complete absence of the protein encoded by an NCL gene usually results in a more severe phenotype than a mutation that results in only a partial loss of function (Mole et al., 2011). Ages of onset of the NCLs are predominantly in early to late childhood, though two rare adult onset forms exist (Haltia, 2006). Collectively the NCLs are possibly the most common form of childhood neurodegenerative disease (Goebel, 1995). The NCLs are predominantly autosomal recessively inherited, with either homozygous or compound heterozygous mutations; one autosomal dominant form exists and is typically transgenerational in presentation (OMIM PS256730).

Before Zeman and Dyken coined the term “neuronal ceroid lipofuscinoses” in 1969 to described the collected group of inherited storage diseases characterized by progressive neuronal and retinal degeneration, this group of diseases had been previously classified as “amaurotic family idiocies” based on their apparent similarity to the prototypic Tay-Sachs disease (Mole et al., 2011). Both G_{M1}-gangliosidosis and G_{M2}-gangliosidosis (Tay-Sachs disease) are characterized by abnormal cerebral sphingolipid profiles as a result of abnormal ganglioside metabolism; however, the NCLs have normal sphingolipid profiles, and they collectively feature the neuronal accumulation of ceroid/lipofuscin type lipopigments (Zeman and Dyken, 1969). Based on this primary difference, along with the autosomal recessive inheritance, progressive vision loss, cognitive decline, loss of motor function, and seizures, variable age of onset, and a more protracted clinical presentation relative to the gangliosidoses, Zeman and Dyken proposed that these diseases were separate from the amaurotic family idiocies.
and should instead be given a more meaningful classification as neuronal ceroid lipofuscinosis (Zeman and Dyken, 1969).

Prior to the advent and use of molecular techniques to determine the underlying genetic basis for the variant forms of NCL, classification was based upon the specific ultrastructural profile and characteristics of the autofluorescent cytoplasmic storage material present within the lysosomal-endosomal compartment (Haltia, 2006; OMIM 204500). In comparison to other lysosomal storage diseases, such as Tay-Sachs disease, in which the major stored material consists mainly of lipids, the storage material in the NCLs is predominantly protein-based (Mole et al., 2011). The different forms can be classified into two categories depending on the protein component of the storage material: those in which subunit c of the mitochondrial ATP synthase is a major constituent and those in which sphingolipid activator proteins A and D are abundant (Haltia, 2006). Storage bodies with large amounts of sphingolipid activator proteins are associated with granular osmiophilic deposits while those in which subunit c is the most common protein component are more variable in appearance and can display a curvilinear, rectilinear, or fingerprint pattern (Haltia, 2006). Each variant form of the disease displays one of these two categories, and those consisting of subunit c may display one, two, or all three of the microscopic patterns (Haltia, 2006). Even within one individual the ultrastructural appearance of the storage material can vary between tissues and organs (Goebel et al., 2004), and the ultrastructure can change with increasing postmortem time. Therefore, any
classification of the NCLs based on existing reports of storage body ultrastructure is unreliable.

1.1.2. CLN2

CLN2 disease (neuronal ceroid lipofuscinosis type 2, previously referred to as classic late infantile NCL and Janský-Bielschowsky disease) is an autosomal recessive lysosomal storage disorder characterized by progressive CNS and retinal degeneration. Symptom onset occurs between 2 to 4 years of age, usually beginning with seizures that are unresponsive to medication and followed by rapid disease progression including motor decline (ataxia) and complete loss of mobility, progressive brain atrophy combined with severe generalized glucose hypometabolism, cognitive regression, myoclonus, progressive vision loss and electroretinogram (ERG) deficiencies beginning at age 4 with complete blindness by 6 to 7 years of age, and impaired swallowing ability (Mole et al., 2011; Goebel et al., 2004). Progressive loss of motor function typically begins at 3 years of age and results in affected individuals usually being bedridden by age 5 to 6, with the disease culminating in premature death by mid-adolescence, 12 to 15 years of age (Mole et al., 2011; Goebel et al., 2004). CLN2 is caused by mutations to the ubiquitously expressed $TPP1$ gene (located on chromosome 11p15.5), which encodes the soluble lysosomal enzyme tripeptidyl peptidase-1 ($TPP1$), an exopeptidase that cleaves N-terminal tripeptides from polypeptide chains (Sleat et al., 1997; Goebel et al., 2004). The absence of or deficiency in functional $TPP1$ results in the accumulation of autofluorescent lysosomal storage material
that displays a distinctive curvilinear profile at the ultrastructural level in neurons throughout the CNS and presents concomitantly with progressive degeneration and loss of function in these tissues. Though natural substrates of TPP1 are unknown, accumulation of the subunit c of mitochondrial ATP synthase (SCMAS) has been observed consistently within the lysosomal storage material of CLN2 subjects, suggesting a causal relationship between the two: the accumulation of SCMAS within the lysosome is caused by its decreased degradation (Kida et al., 2001).

CLN2 is classified as a rare/orphan disease, with an estimated worldwide prevalence of between 0.6 to 0.7 per million inhabitants and an incidence of approximately 0.46 per 100,000 live births (Goebel et al., 2004); however, because of the difficulty in obtaining a proper diagnosis, even in highly developed countries with firmly established medical systems, both the worldwide estimated prevalence and incidence are likely very conservative. Currently no cure is available, although a recently completed clinical trial indicates that enzyme replacement therapy is effective in substantially slowing disease progression (BioMarin presentation at the 2016 WORLD conference).

Studies in mice using recombinant adeno-associated virus (AAV1) injected intracranially have established that the timing of treatment (pre-symptomatic versus post-symptomatic) has significant outcomes on treatment efficacy, with preservation of motor function achieved when treated pre-symptomatically and a more than doubling in survival time versus untreated mice. In contrast, post-symptomatic treatment resulted in limited motor function
recovery and a survival time of roughly 1.4 times that of untreated (Cabrera-Salazar et al., 2007). Similarly, studies in a canine model of CLN2 disease ($TPP1^{-/-}$) utilizing rAAV.caTPP1 administered to the CNS pre-symptomatically showed good efficacy in delaying neuronal symptoms (Katz et al., 2015). In treated dogs, clinical signs of neuronal degeneration were either delayed or never appeared, including impaired hindlimb proprioceptive response, abnormal nystagmus, abnormal pupillary light reflex, cerebellar ataxia, and thoracic limb dysfunction. Furthermore, improvements in cognition were observed in treated dogs along with significant extensions in life span, increasing the mean age of euthanasia from 10.4 months for untreated $TPP1^{-/-}$ dogs to approximately 17.5 months for rAAV.caTPP1-treated dogs.

Given the importance of timing with regards to treatment prognosis and the development of therapeutic interventions, early diagnosis is critical. Early prenatal diagnosis is possible in families with a history of CLN2 disease, as both parents are obligate heterozygotes, and carrier testing for relatives is possible once the disease-causing mutations are known. Barring the addition of CLN2 to newborn blood spot screening or family history, disease diagnosis will be made following symptom onset, either through electron microscope (EM) imaging of the storage materials, enzymatic assay to determine TPP1 activity, or sequencing of the $TPP1$ gene for mutations (Goebel et al., 2004).
1.1.3. Canine Model of CLN2

In 2006, the lab identified a Dachshund that had displayed clinical signs suggestive of a new canine neuronal ceroid lipofuscinosis (NCL) before its eventual death (Awano et al., 2006). Autofluorescent storage bodies were identified in multiple regions of the CNS while ultrastructural analysis of the storage bodies revealed that the contents consisted of the curvilinear forms characteristic of the analogous human CLN2 disease. Subsequent sequence analysis of the canine *TPP1* ortholog from this dog identified a homozygous single nucleotide deletion within exon 4 (c.325delC), resulting in a predicted frame shift after codon 107 in the mRNA and premature stop codon at codon 114. The sire and dam of the affected dog were obtained and were found to be heterozygous for the mutant allele. The heterozygotes were bred, and their first litter produced two additional heterozygotes and two puppies homozygous for the mutant allele. The two homozygotes developed a disease with the clinical presentation of NCL, lacked detectable TPP1 activity, and died at age 10.5 months (Katz et al., 2008). The two heterozygotes were healthy and displayed no adverse symptoms.

A research colony has been established at the University of Missouri through breedings of the heterozygous sire and dam of the initial affected dog, their offspring, and unrelated homozygous normal dogs. The colony is propagated through carrier to carrier breedings as well as occasional male affected to female carrier breedings. The dogs typically do not become sexually mature until about 12 months of age, so most affected dogs cannot be used for
breeding. However enzyme replacement and gene therapy treatments have enabled some affected males to survive well beyond 12 months of age. Semen collected from these dogs has been used for breeding whenever possible. Unrelated homozygous normal dogs are occasionally bred into the colony to prevent a deleterious degree of inbreeding. To further minimize inbreeding, in all matings the lab avoids breeding dogs that have a common ancestor among the parents and grandparents of the dogs being bred. All puppies are genotyped for the *TPP1* mutation using an allelic discrimination assay and with the current colony size on average one affected puppy is generated every month. Heterozygous dogs have not exhibited neurological abnormalities or disease-related health problems throughout the course of the studies (over a period of 5 years).

Studies utilizing these dogs have been used to further characterize the CLN2 disease phenotype in this canine model and to assess possible therapeutics. The CLN2 dog model identified and developed by the lab is being used to better study disease progression to evaluate therapies that can reliably be translated to human disease applications.

1.1.4. **Retinal Degeneration in Canine CLN2**

After the canine CLN2 model was identified by the lab, studies were conducted to determine the extent of the retinal degeneration phenotype (Katz et al., 2008). All dogs were phenotypically normal at 3 months of age, with normal visual behavior and menace response. ERG responses, including b-wave
response stimulated by low intensity flash, scotopic flash response, and photopic single flash and flicker responses, of the affected dog were also not significantly different than those of its unaffected littermates at this time point. By 7 months, however, the affected dog began to show reduced visual capacity, no longer had a menace response, and by 8 months was deemed functionally blind. Low intensity flash stimulated b-wave response was nearly undetectable, and both a-wave and b-wave responses from scotopic stimuli and from photopic stimuli were markedly diminished. At 10 months of age, the affected dog showed increased granularity in the tapetal fundus and vascular attenuation was observed in both the tapetal and nontapetal fundus. The decline in ERG response amplitudes observed at 7 months continued through 8 and 10 months of age, with an electronegative response in place of a b-wave observed under low intensity stimulation, the b-wave failing to reach above baseline under scotopic conditions, no responses under photopic conditions, and decreased and more irregular responses from flicker simulation. Electropositive spikes were observed under both scotopic and photopic conditions. Morphological analysis of the affected dog’s retinas indicated significant cell loss within the photoreceptors and inner nuclear layer in both the superior and inferior retinas compared with the unaffected littermates. Furthermore, irregularity in both shape and size was visible in the photoreceptor outer segments as well as increased intracellular space in the inner and outer segments and a mounded profile of the RPE cells.

Studies conducted by Whiting et al. using the canine TPPI\textsuperscript{−/−} model endeavored to further investigate aspects of CLN2-related retinal degeneration
and potentially identify possible biomarkers to gauge putative treatment effects. One study utilized assessment of the pupillary light reflex (PLR) in both normal control and CLN2-affected dogs to evaluate retinal function (Whiting et al., 2013). No significant difference in baseline pupil diameter was seen between the normal and 8 month or younger affected dogs; however, 10 month old affected dogs had significantly smaller baseline pupil diameter than normal dogs (8.12 ± 0.15 mm versus 6.97 ± 0.40 mm). Furthermore, PLR constriction amplitude was reduced at all time points after 4 months of age in the affected dogs in response to stimuli of 9, 10, 11, and 15 log photons/cm²/s. Additional aspects of the PLR, including latency, constriction velocity, and initial redilation velocity in 10 month old dogs were also significantly reduced relative to normal dogs at stimulus intensities ranging from 8 to 11 log photons/cm²/s. A second study by Whiting et al. quantified the progressive development of retinopathy in the canine CLN2 model (Whiting et al., 2015). Using spectral-domain optical coherence tomography and confocal scanning laser ophthalmoscopy, Whiting confirmed that the multifocal retinal lesions observed in TPP1⁻ dogs were serous retinal detachments and developed a grading system to better quantify the progressive lesion development: grade 1 (less than 15% of retina affected), grade 2 (greater than 15% but less than 30%), and grade 3 (greater than 30%). Average age of lesion onset was 7.5 months, with 23 of 35 dogs in the study developing lesions at some point prior to disease end-stage (approximately 10-11 months of age). Of the 23 dogs that developed lesions, 9 dogs eventually progressed from grade 1 to grade 2, and of those, 3 dogs progressed to grade 3.
1.1.5. Methods of Treatment: Experimental and Clinical

As mentioned previously, no cure exists for CLN2, and until recently all treatments were symptomatic and palliative. Independently, administration of recombinant TPP1 pro-enzyme (enzyme replacement therapy) and AAV2-CAG-TPP1 (direct gene therapy) to the CNS via infusion into the cerebral spinal fluid (CSF) have shown success in significantly ameliorating neuronal degeneration and prolonging life but not in preventing retinal degeneration (Katz et al., 2014; Katz et al., 2015). The therapeutic benefit of the enzyme replacement therapy is dose dependent, which suggests that a sufficiently high, sustained dose may completely prevent the development and progression of neurological symptoms. Based upon these successful results, BioMarin Pharmaceutical Inc. recently completed clinical trials of the enzyme replacement approach and based on the outstanding efficacy of this treatment plans to apply for regulatory approval to market this treatment by mid-2016. Based on the promising results of the lab’s preclinical studies of the direct gene therapy approach (Katz et al., 2015), Spark Therapeutics, Inc. is in the process of applying for regulatory approval to conduct a human clinical trial using this approach to therapy.

BioMarin has developed a recombinant form of human TPP1 named cerliponase alfa, which has been assigned Orphan Drug designation in the United States and European Union, and has completed phase I/II trials in the US and EU to evaluate safety and efficacy of the drug for use in enzyme replacement therapy (BioMarin Pharmaceutical, 2016). Similar to the treatment regimen employed in the canine studies, study participants receive a dose of the
enzyme biweekly via intraventricular delivery for at least 48 weeks. BioMarin recruited participants between 3 and 16 years of age who previously had a diagnosis of CLN2 and presented with mild to moderate disease symptoms. As treatment began prior to disease onset in the foundational canine studies and previous studies in mice have demonstrated that treating before the development of symptoms is critical in the preservation of function (Cabrera-Salazar et al., 2007), the degree the enzyme replacement therapy will delay symptom progression and prolong life is unknown.

Regardless of treatment approach—enzyme replacement therapy or direct gene therapy—based on the lab’s studies with the canine model it appears that neither will prevent the progression of retinal degeneration and vision loss associated with CLN2 disease. While CNS treatment will presumably prevent or at least delay neuronal degeneration in humans similar to the results seen in both canine and mouse models, the replacement TPP1 protein will not be able to cross the blood-retina barrier. Therefore, a directed therapy towards the retina is necessary in order to prevent degeneration, though unfortunately, no such treatment currently exists.

1.2. Enzyme Replacement and Gene Therapy

1.2.1. Current Status

The lab has previously conducted studies to determine whether administration of TPP1 pro-enzyme or AAV2-CAG-TPP1 to the CNS prevents or
delays the disease-related neurodegeneration and related neurological signs in the canine CLN2 model. Widespread distribution of the pro-enzyme into the CNS is achieved through the delivery of the pro-enzyme or vector into the CSF, which circulates throughout and bathes the CNS in its entirety (Sakka et al., 2011; Bulat and Klarica, 2011). In an initial, short-term study, affected dogs were administered 4 bolus injections of recombinant human TPP1 pro-enzyme (rhTPP1) into the cisterna magna at 2-week intervals. The dogs were euthanized 48 hours following the last injection and a range of brain regions were analyzed for active TPP1 enzyme levels and amounts of the disease-typical autofluorescent storage material. TPP1 activity was detected in every brain region examined and in many regions the enzyme activity was a significant fraction of normal levels (Vuillemenot et al., 2011). The amount of storage material in many brain regions was similarly reduced following treatment.

The direct bolus injections performed in the initial study resulted in immune-mediated anaphylaxis. Therefore, subsequent administration of the rhTPP1 pro-enzyme was performed via slow infusion into the CSF through an implanted catheter (Katz et al., 2014). The treatment protocol has been sufficiently optimized such that any detectable adverse immune reaction in response to the recombinant protein is minimal: anti-TPP1 antibodies are detectable in the plasma and CSF, but do not trigger anaphylaxis or inflammation. Using this updated protocol, the lab has administered the pro-enzyme into the CSF in increasing doses at 2-week intervals until the target dosage was achieved. With this treatment approach the lab has achieved
widespread distribution of active rhTPP1 to most brain regions and significant delays in both onset and progression of disease-associated neurological signs and neurodegeneration. Similar results have been achieved through the administration of AAV2-CAG-TPP1 to the CSF of affected dogs at 3 months of age (Katz et al., 2015). However, with this approach to treatment, the dogs suffered from chronic CNS inflammation that had to be treated with a combination of immunosuppressants and steroids. In the enzyme replacement therapy studies, the lab observed that the higher the final dose a dog received the better the outcome, therefore little risk appears to be associated with giving high doses of TPP1 pro-enzyme as long as immunotolerance is induced by starting with a low dose of enzyme and gradually increasing the dose until the target dose is achieved.

Both the enzyme replacement and direct gene therapy studies demonstrate that delivery of the TPP1 pro-enzyme to the CSF allows it to reach most brain regions, where it is then taken up into the brain parenchyma and is activated in the parenchymal cell lysosomes. Accompanying this delivery and subsequent uptake are marked delays in many quantifiers of disease progression in brain structure and function. Similar benefit was not observed with relation to disease-related retinal degeneration or loss of retinal function, but was not unexpected due to the CSF not contacting the retina (Whiting et al., 2013). Based upon the CNS studies, it is anticipated that delivery of the TPP1 pro-enzyme to the retina will result in analogous delays in retinal degeneration.
1.2.2. Vector Selection

Two of the most prominent gene therapy vectors are HIV-1 lentivirus and adeno-associated virus (AAV). The most prominent drawback of lentivectors is that they are subject to insertional mutagenesis in animal and cell-based models (Escors et al., 2011), while AAV has been shown to only integrate within the human genome at a specific locus in chromosome 19, known as AAVS1 (adeno-associated virus integration site 1) (Shakhbazau et al., 2009). In non-human cells, the AAV genome exists episomally within the nucleus of nondividing cells, allowing for long-term expression without risk of mutagenesis (Mi et al., 2009). AAV has no known pathology and low toxicity, further increasing its suitability for use in gene therapy (Shakhbazau et al., 2009). For this reason, the lab has chosen to examine the viability of using AAV mediated gene therapy for the delivery of the TPP1 coding sequence to cells with a lack of functional enzyme. AAV has a large natural tropism, with specific serotypes transducing specific cell types with variable degrees of efficacy; of these, AAV2 is the most well-studied and utilized serotype. Although AAV2 transduces mesenchymal stem cells with low efficiency at low multiplicities of infection, effective transduction is possible with a sufficiently high MOI (Shakhbazau et al., 2009).

1.2.3. In Vivo Versus Ex Vivo

In considering gene therapy for the treatment of CLN2 related retinal degeneration, two different delivery mechanisms are possible: direct injection of the gene therapy vector, either intravitreally or subretinally, or through intravitreal
injection of cells that have been previously transduced with the vector \textit{in vitro}. Both approaches have their own benefits and potential drawbacks that must be considered. With regards to direct injection of the vector (\textit{in vivo}), there is a risk that the treated individual may already have been previously exposed to the gene therapy vector and have developed neutralizing antibodies against it, thereby rendering the treatment ineffective due to poor transduction of cells by the vector (Halbert et al, 2006). Furthermore, additional retreatment may not be possible even in individuals who did not display significant antibody development against the vector due to priming and the possibility of triggering an immune reaction against the vector. Biodistribution of the gene therapy vector beyond the targeted area may also occur and must be taken into account; injection of recombinant AAV (rAAV) into either the subretinal space or vitreous in rats, primates, and dogs has been seen to result in detection of rAAV in the retina and optic nerve of the injected eye but not the contralateral retina or optic nerve, and in the brains of intravitreally injected animals (Provost et al., 2005). Additionally, rAAV genomes have occasionally been identified in lymph nodes and peripheral blood mononuclear cells independent of the administration location (Provost et al., 2005). It may also be possible that the transduction of specialized cells within the retina by AAV gene therapy vectors could have a negative effect on their homeostatic metabolism: the transduction of cells could convert them into essentially transgene expression factories, thereby interfering with their normal essential function within the retina. Finally, the location of vector administration can also influence which cells are efficiently transduced: subretinal delivery of
AAV4 transduces retinal pigmented epithelium (RPE) cells, AAV2 and AAV5 results in transduction of both the RPE and photoreceptors, while retinal ganglion cells are transduced following intravitreal injection (Provost et al., 2005).

Unlike the direct in vivo gene therapy, ex vivo gene therapy is a more indirect method of treatment: transducing autologously derived cells with the gene therapy vector in vitro and then injecting the cells themselves into the patient once they are seen to express the transgene in question. This method has several advantages over the in vivo gene therapy approach, mainly that by using the patient’s own cells as the delivery method for the transgene expressed protein and by performing the transduction in vitro, the risk of an immune response against either the cells or the gene therapy vector is greatly reduced. The ex vivo method is not without certain drawbacks, however: there is a possibility that the implanted cells may invade and disrupt the tissues surrounding the injection site or even potentially form a tumor. The risk of tumor growth though can be mediated by the choice of gene therapy vector; insertional mutagenesis is less of a risk with AAV than with the lentiviruses, as it does not insert randomly into the genome (Shakhbazau et al., 2009).

The relatively wide biodistribution of rAAV following intravitreal injection into the optic nerve and the visual pathway regions of the brain observed in dogs is of particular note; while the presence of exogenous TPP1 has not been observed to have any deleterious effects within the central nervous system, and may in fact be a positive side effect of a potential retinal therapy, the non-tissue-specificity of rAAV vectors resulting in expression of transgenes outside of the
targeted tissue is problematic. Both methods have a potential hazard in that in the case of a complete knockout of a gene and a resulting total lack of protein produced, the immune system would not recognize the protein produced by the vector as self and could potentially attack and degrade it. Given though that the presence of these proteins is necessary for normal cellular function, a potential immune response against the protein must be an accepted possibility if a treatment is to be found.

Anti-TPP1 neutralizing antibodies were observed in $TPP1^{-/-}$ dogs that received rAAV.caTPP1 direct gene therapy in a study evaluating the efficacy of gene therapy administered to the CNS as a means of ameliorating the CLN2 CNS phenotype (Katz et al., 2015). Five days post-injection, treated dogs had up to 30-fold greater CSF TPP1 concentrations relative to normal control dogs. However, by approximately two months post-injection, TPP1 levels had returned to background, with subsequent analysis indicating a rise in neutralizing antibodies. Treated dogs were administered mycophenolate mofetil, an immunosuppressant, 44 and 33 days after vector administration. No recovery of TPP1 activity was observed in the dogs administered starting at 44 days, while in those dogs beginning at 33 days lower antibody titers were observed along with increases in TPP1 activity. All other dogs included in the study were started on mycophenolate at five days pre-injection of the rAAV.caTPP1 vector and subsequently did not develop anti-TPP1 antibodies (Katz et al., 2015).

Similar to the development of anti-TPP1 neutralizing antibodies observed in the direct gene therapy study, an anaphylactic reaction was also observed
during the slow infusion of recombinant human TPP1 (rhTPP1) in a catheter-mediated enzyme replacement study conducted using the same $TPP1^{-/-}$ dog model (Katz et al., 2014). The treated dog received two initial 48 mg doses of rhTPP1, each administered over a 4 hour period two weeks apart, with no adverse reaction. During the third infusion, the dog had an anaphylactic response against the enzyme and treatment was halted. The next subsequent dose was reduced to 2 mg rhTPP1 and was gradually increased over the course of 15 additional biweekly infusions until the original 48 mg dose had been achieved. All subsequent infusions remained at 48 mg and were tolerated without additional response (Katz et al., 2014).

1.2.4. Amenability for Use in CLN2

As with other soluble lysosomal enzymes, TPP1 is synthesized in an initially catalytically inactive pro-enzyme form on the rough endoplasmic reticulum and is post-translationally modified through the glycosylation of multiple asparagine residues. The pro-TPP1-glycoprotein is then exported to the Golgi apparatus where the oligosaccharides are converted to mannose-6-phosphates (M6P). The M6P moiety is utilized as a targeting signal for the lysosomal acid hydrolase precursors. Pro-TPP1-M6P is then trafficked to the lysosomes via M6P-mediated signaling, upon which it is autoactivated by proteolysis within the acidic lumen (Chen et al., 2009; Dahms et al., 1989; Guhaniyogi et al., 2009; Grubb et al., 2010). The TPP1 pro-enzyme can reach the lysosome not only via intracellular trafficking but also through intercellular exchange. Many cell types,
including neurons, express extracellular-facing, membrane-bound M6P receptors (MPRs). M6P-tagged proteins, including TPP1, will bind to these membrane-bound MPRs, be endocytosed, and then be transported to the lysosomes through the endosomal system (Dahms et al., 1989). As also occurs in delivery through intracellular trafficking, the endocytosed pro-enzyme is activated upon reaching the acidic environment of the lysosome (Guhaniyogi et al., 2009). The M6P tag is preserved on the mature TPP1 enzyme, so mature TPP1 can also reach the lysosome if supplied exogenously (Grubb et al., 2010; Dell’Angelica et al., 2001).

The lab has previously found that administration of recombinant human TPP1 pro-enzyme or AAV2-TPP1 into the cerebrospinal fluid (CSF) results in a dramatic delay in onset and severity of disease symptoms within the CNS and a prolongation of the disease course, as well as substantially extends the life span of CLN2 affected dogs (Katz et al., 2014; Katz et al., 2015). This illustrates the efficacy of an enzyme replacement approach with regards to a lack of functional TPP1 within the cell; if supplied exogenously, regardless of delivery method into the body, replacement enzyme can rescue the disease phenotype and restore normal or near-normal cell function.
1.3. Large Animal Models

1.3.1. Murine Versus Canine for Translational Research

While NCL disease mouse models exist, including a CLN2 model, such models have inherent limitations beyond early stage disease progression and pathogenesis. In the development of a targeted disruption CLN2 mouse model, Sleat et al. noted that median survival time fluctuated significantly depending on the background into which the mutation was induced, with median survival of 138 days in C57BL/6:129Sv mice and 164 days in isogenic 129Sv (Sleat et al., 2004). This background-dependent variance in median survival time may suggest that a strain-specific modifier exists that can modulate the CLN2 phenotype in the mouse model (Sleat et al., 2004). Further characterization of the CLN2 mouse model demonstrated a preservation of the retina and photoreceptors, though the presence of autofluorescent storage material was observed in some cells (Sleat et al., 2004). These limitations, particularly the lack of retinal degeneration observed, combined with the gross anatomical differences in structure between the human and mouse eye, make the mouse model less than ideal when assessing possible therapeutic interventions with regards to the eye and retina. In comparison, large animal models, such as dogs, are better suited to such studies due to the greater similarity in eye structure, though dogs do lack a macula and fovea, longer life expectancy allowing for long term analysis of therapeutic efficacy throughout the disease course, and a clinical progression, specifically, the progressive loss of retinal function and the loss of cells within the
retina as well as disruption of its structure, more closely resembling that in humans (Mole et al., 2011; Katz et al., 2008). As such, while approaches initially developed in the mouse models are useful as pilot studies and for determining overall feasibility and refinement, as in the case of gene transfer and enzyme replacement approaches tested in the CLN2 mouse as well as the determination of the importance of timing with regard to therapeutic intervention and the preservation of function and survival, and said models can be used to identify quantitative landmarks of disease progression with which to measure treatment efficacy (Mole et al., 2011; Passini et al., 2006; Sondhi et al., 2011; Cabrera-Salazar et al., 2007; Sondhi et al., 2008; Chang et al., 2008; Chen et al., 2009), large animal models are necessary for the development of translatable approaches for clinical studies.

1.4. Mesenchymal Stem Cells

1.4.1. Isolation

Mesenchymal stem cells (MSCs) are a multipotent cell population canonically found within bone marrow and adipose tissue, while recent studies indicate that MSCs may be present in vascularized tissue throughout the body as well as in umbilical cord, umbilical cord blood, and placenta (Lv et al., 2014). MSCs facilitate the regeneration of mesenchymal tissues including bone, cartilage, tendon, and ligament as well as muscle, adipose (only from cells derived from bone marrow and adipose tissue; Kern et al., 2006), and stroma.
Culturing cells isolated from the bone marrow in the presence of fetal bovine serum (FBS) have been seen to select for the expansion of a marrow cell population with MSC potential (Pittenger et al., 1999). Mesenchymal cells can be identified by their ability to adhere to substrate and to proliferate with a well-spread morphology and the formation of symmetric colonies following initial plating (Pittenger et al., 1999). Following initial plating, hematopoietic stem cells and other nonadherent cells present in a bone marrow aspirate are removed following several washes and changes of culture media over the course of 1 to 2 days. By passages 1 and 2, the cells typically consist of a single phenotypic population, with the use of trypsin to detach the cells from the culture substrate having no adverse effect (Pittenger et al., 1999).

1.4.2. Characteristics In Vitro

Flow cytometric analysis of expressed surface antigens from expanded attached mesenchymal cells show that the cells are uniformly positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a, and CD124, among other surface proteins (Pittenger et al., 1999). Previous studies showed that no subpopulations of mesenchymal cells derived from the marrow could be discerned morphologically, through fluorescence cytometry, or through using multiple antibodies, and that the cells were negative for markers of a hematopoietic lineage (CD14, CD43, and CD45) and that furthermore no hematopoietic cells were identified in culture (Pittenger et al., 1999). Cultured
cells do not differentiate spontaneously during expansion and maintain normal karyotype and telomerase activity after multiple passages (Pittenger et al., 1999).

1.4.3. Potential for Use in Biologic Delivery

Several studies in mice have shown that both murine and human MSCs can survive following intraventricular injection as well as injection into discrete brain tissues and are capable of migrating beyond the initial injection sites throughout the brain (Kopen et al., 1999; Jin et al., 2002; Kim et al., 2010). In a mouse knockout model (ASMKO) of types A and B Niemann-Pick disease (NPD), murine MSCs transduced with a retroviral vector to overexpress acid sphingomyelinase and injected into the cerebellum and hippocampus were seen to survive post-injection for at least 6 months, significantly increase survival in treated mice versus untreated controls, and delay Purkinje cell loss (Jin et al., 2002).

In a null PPT1 (encodes the soluble lysosomal enzyme palmitoyl protein thioesterase-1) mouse model in which the retina initially develops normally but then undergoes progressive degeneration, MSCs harvested from eGFP-expressing normal C57BL/6 J mice were implanted intravitreally (Tracy et al., 2015). The mutant mice tolerated the intravitreal injections well, with the implanted cells forming stable sheaths within the vitreous and displayed neither proliferation nor cell loss over the 16 week evaluation period. Furthermore, there was no evidence of the cells migrating towards or into the retina. In a pilot study utilizing a canine model of CLN2 disease (null TPP1 mutation), MSCs were
cultured from bone marrow aspirates and transduced with an AAV2 vectors in vitro directing the overexpression of GFP or TPP1. GFP-transduced cells kept at confluency without further passaging post-transduction were seen to maintain high levels of fluorescence for at least 70 days in vitro. Media from TPP1-transduced cells was collected and analyzed for active TPP1 enzyme activity, with an estimated extracellular release of enzyme by the cells in vitro of 3 to 5 pg per cell per 24 hours (Tracy et al., 2015).

1.5. Electroretinography

Full-field electroretinogram (ERG) is a widely accepted clinical electrophysiological test of retinal function that allows for reproducible response recordings between individual laboratories. The standard ERG series consists of six protocols that provoke responses from different cell types within the retina: dark-adapted 0.01 (0.01 photopic cd·s·m⁻²/0.025 scotopic cd·s·m⁻²) ERG, dark-adapted 3 (3 photopic cd·s·m⁻²/7.5 scotopic cd·s·m⁻²) ERG, dark-adapted 10 (10 photopic cd·s·m⁻²/25 scotopic cd·s·m⁻²) ERG, dark-adapted oscillatory potentials, light-adapted 3 (0.01 photopic cd·s·m⁻²/0.025 scotopic cd·s·m⁻²) ERG, and light-adapted 30 Hz flicker ERG (Ekesten et al., 2013; McCulloch et al., 2014). In order, they elicit a rod-system response, combined rod and cone system responses, combined rod and cone system responses to a stronger flash, a single-flash cone response, and a cone-system response, respectively. The protocols are named with reference to the strength of the flash stimulus (in cd·s·m⁻²) and whether the patient has either been dark-adapted or light-adapted.
prior to the administration of the stimulus. In the first three and fifth single-flash ERG protocols (dark-adapted 0.01, 3, and 10 ERG and light-adapted 3 ERG), the a-wave and b-wave amplitudes, and peak time are recorded and measured when present. The a-wave amplitude is measured from the pre-stimulus baseline to the trough of the a-wave while the b-wave amplitude is measured from the trough of the a-wave to the b-wave peak; peak time corresponds from the flash to the wave peak. Oscillatory potentials generally consist of three positive peaks and a fourth smaller peak, the typical presence and normality of which is a sufficient metric. For the flicker ERG, the amplitude is measured from the trough to peak of a wave and the peak time is measured from the midpoint of the stimulus to the following peak (Ekesten et al., 2013; McCulloch et al., 2014).

In both human and canine CLN2, ERG responses progressively deteriorate over time, concomitant with progressive degeneration of the retina (Weleber, 1998; Goebel et al., 2004; Katz et al., 2008). Of the six standard ERG protocols, of particular interest is the dark-adapted 10 ERG, as it elicits a strong combined response from both photoreceptor systems with predominant a- and b-waves, and provides greater distinction of electronegative waveforms such as those seen following reduction in b-wave amplitude and as observed in the canine CLN2 model (McCulloch et al., 2014, Katz et al., 2008).

1.6. Study Objectives

While other treatment modalities may be adaptable to address the retinal degeneration observed in CLN2, such as enzyme replacement and direct gene
therapy, they are not ideal. The rapid turnover of the TPP1 enzyme once inside the body combined with non-specific cell transduction by gene therapy vectors, the possibility of deleterious insertion of the vector within the genome, the potential of an immune response against the vector if repeat transductions are required for efficacy, and the potential for chronic inflammation in response to direct gene therapy necessitates the use of an alternative therapeutic approach. 

*Ex vivo* gene therapy may be a potential alternative; cells are autologously derived thereby limiting the chance of an immune response, transduction of cells occurs *in vitro* and any vector proteins have been washed away or degraded before the cells are injected into the subject, and based on previous studies, once injected the transduced cells should remain in place and continue to produce the transgene for long periods of time (Tracy et al., 2015). If successful, this *ex vivo* gene therapy approach may be adapted not only to treat retinal degeneration and blindness in children affected by CLN2, but as a therapeutic architecture for the use in sustained delivery of other protein and biologic medical products to the eye and retina in other ophthalmic disorders.

The specific aims for this study are as follows:

1. Characterize the behavior of AAV2-GFP transduced canine mesenchymal stem cells (MSCs) *in vitro*.

2. Determine the feasibility of injecting transduced MSCs intravitreally and characterize behavior of cells *in vivo*. 
3. Evaluate the efficacy of AAV2-CAG-TPP1 transduced MSCs to delay disease-related retinal degeneration in a canine model of CLN2 using ophthalmic biomarkers.

4. Evaluate the efficacy of AAV2-CAG-TPP1 transduced MSCs to delay disease-related central nervous system degeneration in a canine model of CLN2 using neurologic biomarkers.
CHAPTER 2

TRANSDUCTION AND TRANSPLANTATION OF CANINE MESENCHYMAL STEM CELLS

2.1. Introduction

The development of therapeutic applications utilizing stem cells, and specifically in applications involving the regeneration of tissue that has been damaged via injury or disease, has seen a substantial increase in focus over recent years (Kim and de Vellis, 2009; Jung et al., 2011; Bhasin et al., 2012; Fisher et al., 2013). Of particular interest are those tissues that are not otherwise amenable to treatment through systemic or topical administration of a therapeutic. The human eye, and the retina specifically, is one such area of interest as its degeneration is associated with multiple disease types among which include age-related macular degeneration, macular dystrophies and inherited macular degeneration, and inherited retinal degenerations such as Leber congenital amaurosis, retinitis pigmentosa, achromatopsia, and the neuronal ceroid lipofuscinoses (Mole et al., 2011; Tibbets et al., 2012; Ng et al., 2014). This last group, the neuronal ceroid lipofuscinoses (NCLs), is particularly interesting as several of the disease variants including CLN1, CLN2, CLN5, CLN10, and CLN13 result from mutations to genes that encode soluble proteins (OMIM PS256730). If modified to overexpress the functional genes for which there are protein deficiencies in these diseases, genetically modified stem cells could provide a long term source of functional protein to the retina following
implantation into the ocular vitreous, thereby delaying or preventing retinal degeneration and preserving visual function.

We hypothesized that transgenic autologous stem cells injected into the vitreous of dogs (termed here as "ex vivo gene therapy") could serve as a sustained delivery mechanism for soluble protein therapeutics to the retina, and that cells injected intravitreally in this fashion would not have any deleterious effects on the retina itself due to their presence and would continue to express a reporter transgene for long periods of time in vivo. In order to determine whether an ex vivo gene therapy approach for the treatment of NCL-related retinal degeneration would be viable, studies were necessary to gauge various safety and logistical aspects of the putative therapeutic approach: the amenability of the chosen stem cell type (bone-marrow derived mesenchymal stem cells, MSCs, in this specific application) for transduction by the chosen gene therapy vector (adeno-associated virus, serotype 2, AAV2); whether the transduced MSCs are able to survive in vivo; if the transduced MSCs are able to maintain transgene expression in vivo and for how long; what, if any, deleterious effects the MSCs may have on the retina and the eye; and if cells proliferate after implantation. The results of these studies would then be used to inform the feasibility of the ex vivo gene therapy approach as originally conceived, whether any modifications to the approach would be necessary, and ultimately whether such an approach would be worthwhile in the further investigation as a potential therapy for NCL-related retinal degeneration and degeneration of the central nervous system.
2.2. Materials and Methods

2.2.1. Animals

Studies employed affected purpose-bred long-haired miniature Dachshunds (n=6). Affected dogs were homozygous for a one nucleotide deletion (c.325delC) in exon 4 of the *TPP1* gene (*TPP1*<sup>-/-</sup>) that results in a complete absence of functional TPP1 enzyme (Awano et al., 2006). Puppies were genotyped within several weeks of birth at the *TPP1* locus using an allelic discrimination assay that distinguishes between the normal and mutant alleles (Awano et al., 2006). Dogs were housed and bred in AAALAC-accredited facilities maintained by the University of Missouri Office of Animal Resources. Dogs were maintained on a 12:12 daily light cycle and were socialized daily in addition to receiving routine husbandry care. All studies were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Missouri Animal Care and Use Committee (ACUC).

2.2.2. Ophthalmic Examinations

Prior to their inclusion in the study, all dogs received a complete ophthalmic examination at 10-12 weeks of age, before the onset of any signs of retinal or neurological impairment due to CLN2 in the affected dogs. Dogs with evidence of compromised vision or any ophthalmic abnormalities deemed threatening to vision were excluded from the studies. All dogs received ophthalmic
examinations monthly throughout the study. Examination included visually-mediated behavioral assessment and slit lamp biomicroscopy (SL14; Kowa Co. Ltd., Tokyo, Japan). Pupils were dilated with a short acting mydriatic (tropicamide 1%; Alcon, Fort Worth, TX), and indirect ophthalmoscopy (12500, Welch Allyn Inc., Skaneateles Falls, NY, USA) was performed. Fundus photographs (NM-100; Nidek Co. Ltd., Freemont, CA) were taken following examination and archived electronically. Photos were reviewed to evaluate for abnormalities in retinal appearance.

2.2.3. Bone Marrow Isolation

Bone marrow aspirates were collected from each dog at approximately 2.5-3.5 months of age. Dogs were given intramuscular injections of dexmedetomidine hydrochloride (5 µg/kg) morphine (0.5 mg/kg) followed by intravenous Propofol administration to effect (1.49 ± 0.59 mg/kg (mean ± SD)); PropoFlo 28, Abbott Laboratories, Abbott Park, IL). Dogs were intubated with a cuffed endotracheal tube and restraint maintained with isoflurane (1.5% vaporizer setting; Terrell, Piramal Healthcare, Boise, ID) in oxygen during the aspiration process. A local intramuscular injection of lidocaine was administered just prior to aspirate collection.

Bone marrow was collected from the humerus using sterile procedures. The hair on the forelimb over the proximal end of the humerus was clipped, shaved, and surgically prepared. The proximal portion of the greater tubercle was palpated and a small incision over this structure was made using a #15
surgical blade. An 11-gauge 10 cm Jamshidi needle was introduced through the incision and pushed into contact with the lateral portion of the greater tubercle in the fossa where the lateral glenohumeral ligament attaches. The needle was passed into the bone using a drilling motion aiming the needle at an angle to pass down the medullary cavity. When the needle had penetrated fully through the cortex, the needle was advanced down the medullary canal for a short distance and the stylet was removed. A 10 mL syringe contained 0.2 mL preservative-free heparin was attached to the needle and approximately 5 mL of marrow was withdrawn while the needle was continuously rotated to prevent possible clot formation. The needle was then withdrawn and the marrow was expelled into 3 mL of sterile 1X Minimum Essential Medium (MEM) Alpha (Thermo Scientific cat. No. SH30265.02).

2.2.4. Cell Culture

The bone marrow preparation was vortexed for 30 seconds and then spun for 10 minutes at 1591 xg (Unico Powerspin centrifuge, “Blood” preset) to pellet the cells. Using sterile procedures, the supernatant was removed by vacuum suction and discarded and the cells were suspended in 5 mL of fresh stem cell culture medium (SCCM), consisting of 1X MEM alpha, 20% fetal bovine serum (Thermo Scientific cat. No. SH30088.03/Gibco 16000-077), and 1X penicillin/streptomycin (Thermo Scientific cat. No. SV30010). The cell suspensions were plated onto sterile 100 mm • 20 mm culture plates and additional SCCM was added to each plate to bring the total volume to 10 mL.
The cells were then placed in a sterile incubator at 37°C with 5% CO₂. After 24 hours the medium including any non-adherent cells was drawn off, the plates were washed with sterile 1X phosphate-buffered saline (PBS) (diluted from 10X solution, Fisher BioReagents cat. No. BP399.4), and 10 mL of fresh SCCM was added to the plates. The cells were washed again after an additional 24 hours to remove any remaining red blood cells and fresh SCCM was added.

Every 2 to 4 days the spent SCCM was removed from the cultures and replaced with fresh SCCM. After the cells reached confluence, the medium was removed, the cells were washed with PBS, and then incubated for 6 to 15 minutes at 37°C in freshly-thawed 0.05% trypsin in PBS. Plates were briefly removed from the incubator after 6 and 12 minutes and subjected to gentle physical agitation in order to facilitate cell detachment. The trypsin was inactivated by the addition of a sufficient volume of SCCM (4 times the volume of trypsin solution added to each plate). The cell suspension was then replated at 50% confluence and the cells were incubated and washed regularly until again confluent. The cells that remained attached to the plate at this point were designated mesenchymal stem cells (MSCs) (Pittenger et al., 1999).

2.2.5. Transduction of MSCs

After reaching either passage 3 or 4, the cells were either transduced directly while attached to plates or harvested from the plates as described in Section 2.2.4 and transduced in suspension. Cells from dogs 1-5 were transduced in suspension as the transduction protocol was still being optimized
during the study. Cells from dog 6 were transduced directly was attached to the culture plates as this was more efficient and did not result in an appreciable difference in rate of transduction. The concentration of cells on each plate was estimated via density (numbers of cells per 1 mm² then extrapolated to available surface area on plate). If transduced in suspension, the cells were pelleted after trypsinization and resuspended in SCCM prior to treatment with the transduction vector. The resulting cell suspensions were then transduced with an AAV2-CMV (n=3) or AAV2-CAG (n=3) vector backbone containing a GFP-expression construct (AAV2-CMV-GFP: SignaGen cat. No. SL100812; AAV2-CAG-GFP: SignaGen cat. No. SL00816). The frozen vector was thawed and an aliquot was added to the cell suspension to achieve a multiplicity of infection (MOI) of 50,000 for AAV2-CMV-GFP or AAV2-CAG-GFP and the suspension was then gently mixed. The suspension was then plated and incubated for approximately 96 hours at 37°C in 5% CO₂. If transduced while plated, plates were washed with PBS and then the vector was added to a volume of SCCM and then added to the plates, with additional media added to reach either a total of 10 mL (for 100 mm • 20 mm plates) or 25 mL (for 150 mm • 25 mm plates). Regardless of transduction method, after 96 hours the medium containing the vectors was drawn off and replaced with fresh SCCM and the cells were cultured in this medium until used for implantation.
2.2.6. *In Vitro* Examination

The cells transduced with the AAV2-GFP vectors were evaluated for *GFP* expression using a Leica DMI 600B inverted microscope equipped for epi-fluorescence and phase contrast imaging. Adherent cells were examined with both phase contrast and fluorescent imaging modes to assess the proportions of cells that expressed *GFP*. Fluorescent images were acquired using Leica filter cube L5 ET.

2.2.7. Intravitreal Injection of cMSCs

The transduced MSCs were implanted into the vitreous of the same dog from which the cells were derived. Practical adaptations to the implantation protocol were made after initial testing: for earlier dogs, implantation was performed approximately 5 to 7 weeks after bone marrow aspiration, at approximately 17 to 22 weeks of age, while for later dogs, implantation was performed approximately 2 to 3 weeks after bone marrow aspiration, at approximately 14 weeks of age. Starting 1 week prior to the planned injection, cyclosporine was administered at 25 mg BID. Cyclosporine administration continued at this dose until the dog reached a weight of 4 kg after which the dose was increased to 35 mg BID. Cyclosporine administration at this dose was continued until the dog was euthanized. On the day of injection the cells were harvested from the culture plates as described previously for cell passaging (Section 2.2.4), pelleted, and then resuspended in either MEM alpha or SCCM at concentrations of 1, 2.63, 5, 7.5, 12.5, and 40 million cells per mL and either
injected immediately or held at 37°C for 60 to 90 minutes. Starting 1.5 hours prior to the planned injection, a drop of prednisone acetate 1% ophthalmic suspension and a drop of topicamide 1% ophthalmic solution were administered to the corneal surface every 10 minutes for 30 minutes. Just prior to the injection procedure, 0.2 mg/kg dexamethasone (2 mg/mL) was administered intravenously. The dogs were placed under general anesthesia as described previously for the bone marrow aspirations (Section 2.2.3) and maintained on a ventilator. The eyes were washed with dilute betadine. The tube containing the cell suspension was inverted gently several times and the suspension was then drawn into a sterile 1 mL syringe with a 1 inch 27-gauge needle. With or without the aid of an operating microscope and a Machemer Magnifying Vitrectomy lens (Ocular Instruments, Bellevue, WA), 160 to 300 µL of the cell suspension was injected into the vitreous with the needle entry point 7 mm posterior to the limbus and needle trajectory pointed towards the optic nerve, with the injection made from the top of the eye. Immediately following the procedure 2 mg of triamcinolone acetonide was injected sub-Tenon’s. Prednisolone acetate 1% ophthalmic suspension was administered twice daily for one week following the procedure and tropicamide 1% ophthalmic solution was administered once daily (as needed) for one week following the procedure. After one week, 2.2 mg/kg Carprofen PO BID was administered if uveitis was observed.
2.2.8. Optical Coherence Tomography

Immediately and then one week after injection, eyes were imaged with funduscopy and with a Spectralis HRA/OCT imaging system (Heidelberg Engineering, Heidelberg, Germany) in fluorescence, spectral domain optical coherence tomography (sdOCT), and scanning laser ophthalmoscopy (cSLO) modes. Injected eyes were examined with combined cSLO and sdOCT. All images were obtained with the dog under general anesthesia. Wide-angle cSLO fundus images were obtained using a 55° lens and sdOCT scans were obtained with a 30° lens as a series of parallel linear scans of 40-50 sequential B-scans each separated by 200 µm in both the superior and inferior retina.

2.2.9. Euthanasia and Necropsy

Dogs were sedated with dexmedetomidine and euthanized with Fatal Plus (sodium pentobarbital). The eyes were enucleated immediately and placed in either a fixative consisting of 3.5% paraformaldehyde, 0.075% glutaraldehyde, 0.12 M Na-cacodylate, 0.5 mM CaCl₂ (pH 7.4) (for fluorescence microscopy) or 2.5% glutaraldehyde, 0.1 M Na-cacodylate (for histopathological examination). The corneas, irises, and lenses were removed and the remainder of each eye was incubated in the fixative with gentle agitation at room temperature. For fluorescence microscopy, the fixative was replaced with a solution of 0.17 M Na-cacodylate (pH 7.4) after approximately 24 hours. Eyes to be used for histopathological examination were kept in 2.5% glutaraldehyde fixative until being processed for microscopic examination.
2.2.10. Light Microscopy

Segments of glutaraldehyde-fixed retinas were examined using conventional histological techniques. After incubation of the tissues in the fixative for at least 24 hours, the eyecups were dissected to obtain specific regions of the retina which were then washed in cacodylate buffer, post-fixed with 1% OsO$_4$, and embedded in epoxy resin. Sections of the embedded samples were cut and stained with toluidine blue. The sections were examined with transmitted light microscopy.

2.2.11. Fluorescence Microscopy

The eyecup was examined with a Leica MZFLIII microscope with epi-illumination from a xenon lamp and using a Leica GFP filter set. Clusters of GFP-expressing cells in the vitreous large enough to visualize were dissected from the eyecups along with the surrounding vitreous and transferred to vials containing cacodylate buffer. The cell clusters were then photographed with the MZFLIII microscope.

Eyecups were dissected and processed for cryostat sectioning. Segments of the eyecups were incubated sequentially in 10% sucrose, 20% sucrose, 1:1 20% sucrose:TissueTek, and pure TissueTek. The samples were then incubated in embedding molds filled with TissueTek for 40 minutes and then frozen on dry ice. Sections of the frozen samples were cut with a Microm H525 (Microm International, Waldorf, Germany) cryostat at a thickness of 8 µm. The sections were mounted on SuperFrost slides (Fisher Scientific) in 0.17 M Na-cacodylate
buffer and examined with a Zeiss Axiophot microscope using epi-illumination from a xenon lamp and a Chroma NC322124 filter set optimized for visualization of GFP fluorescence (Chroma Technology, Rockingham, VT).

2.3. Results

2.3.1. Isolation of Canine MSCs and Growth In Vitro

Cells from bone marrow aspirates were allowed to attach to 100 mm • 20 mm plates for a period of 48 hours, during which the plates were washed repeatedly at 24 and 48 hours post-plating with 1X PBS to remove non-adherent cells, the majority of which were red blood cells. The plates were cleared of any remaining non-adherent cells after the 48 hour washes, and the remaining cells were identified as mesenchymal stem cells (MSCs) by their morphology and adherence to plastic (Williams and Hare 2011; Pittenger et al., 1999). Cells were passaged from the initial plating (passage 0, P0) to P1 after they reached initial confluence (here described as the formation of symmetric colonies spanning the entirety of the available growth surface), 2-6 days in SCCM media. During the P0-P1 passage, the initial 100 mm • 20 mm plates were expanded to 150 mm • 25 mm plates at a 1:1 expansion and were maintained on these plates through subsequent passages at a 1:2 expansion. Time between subsequent passages (P2 and P3) varied between dogs, with some cell populations reaching P2 and P3 confluency after 24-48 hours following the previous passage, while others grew more slowly between passages and required 3-5 days to reach confluency.
Regardless of rate of growth, all cells displayed typical MSC morphology and cell morphology remained indicative of an MSC lineage through repeated passage.

### 2.3.2. Transduction of cMSCs

Canine MSCs were transduced with rAAV2-CMV or rAAV2-CAG vectors direction the overexpression of GFP through the addition of the vector to SCCM and then added to each plate (Figure 2-1). The inoculated media was left on the plates for 96 hours, after which it was replaced with fresh SCCM. Initial GFP expression was detectable 96 hours (rAAV2-CAG) to 120 hours (rAAV2-CMV) post-transduction and increased in intensity over time, reaching a stable high level of intensity approximately 5 (rAAV2-CAG) to 7 days (rAAV2-CMV) after transduction. GFP expression remained stable for at least two passages post-transduction in vitro. P4 and P5 transduced cells kept at confluency without additional passaging maintained high levels of fluorescence for at least 70 days in vitro. Based on visual examination, a high percentage of observable cells (near 100%) displayed some level of GFP fluorescence in vitro, with similar transduction efficiency observed at both 10,000 and 50,000 MOI (Figure 2-2).
Figure 2-1: Recombinant adeno-associated vector, serotype 2, backbones utilized in the transduction of cMSCs. Vectors consisted of flanking long terminal repeats (LTRs), a promoter (CAG/CMV), GFP cDNA, and a polyA tail.

Figure 2-2: Fluorescence (A), phase contrast (B), and merged micrographs (C) of rAAV2-GFP transduced cMSCs in vitro cultured from aspirated canine humerus bone marrow cells. GFP expression was maintained for at least 8 weeks in culture. Cells were photographed two weeks post-transduction. Untransduced cells were examined under fluorescence were not seen to display appreciable background fluorescence.

2.3.3. Intravitreal Injection of Transduced cMSCs

Autologous P3 and P4 AAV2-CAG-GFP and AAV2-CMV-GFP transduced MSCs were injected intravitreally at approximately 14 weeks of age at concentrations ranging from 1, 2.63, 5, 7.5, 12.5, and 40 million cells per mL and
were either injected immediately following detachment and collection or held at 37°C for 60 to 90 minutes prior to injection. All cell suspensions were injected into the vitreous with the needle entry point 7 mm posterior to the limbus and needle trajectory pointed towards the optic nerve, with the injection made from the top of the eye (Figure 2-3). The initial dog developed uveitis in its treated eye. Because of this, the injection protocol was modified for all subsequent dogs receiving injections: 2 mg of triamcinolone acetonide was injected sub-Tenon’s immediately following the procedure, prednisolone acetate 1% ophthalmic suspension was administered twice daily for one week following the procedure, tropicamide 1% ophthalmic solution was administered once daily (as needed) for one week following the procedure, and after one week, 2.2 mg/kg Carprofen PO BID was administered if uveitis was observed. With these modifications, uveitis was controlled or absent in the other dogs. In 3 out of 10 injections performed, the retina was touched or poked by the injection needle, though these injuries occurred in earlier dogs studied and can be explained by surgeon error and unfamiliarity with the injection technique. The remaining 7 injections were conducted without incident.
Figure 2-3: Cartoon illustration of the injection of the GFP-transduced cMSCs into the ocular vitreous of a canine eye (left) and the hypothesized formation of a stable sheath or clump of cMSCs located just superior to the retina (right).

2.3.4. OCT

For each of the dogs included in the study, AAV2-CAG-GFP or AAV2-CMV-GFP transduced MSCs were injected into one eye or both eyes, save for one dog which received a second injection of cells into the same eye that was originally injected. Optical coherence tomography (OCT) imaging was used to locate or attempt to locate the cells at various time points post-injection as to monitor the survival of the cells *in vivo* and to ascertain whether the cells were migrating or expanding. In all cases, cells were visible immediately following injection; however, the visibility of the cells via OCT was intermittent among the dogs at future time points. For the first dog examined (one injection, OS), cells remained visible at 7 days post-injection, with one large collection of cells visible...
Cells remained visible in the same location at 21 and 42 days post-injection. The dog was euthanized at 50 days post-injection for examination of the intraocular distribution and morphology of the injected cells. The second dog received injections is both eyes (OS and OD) and cells were visible at 5 days post-injection in both. Cells remained visible at 33 and 63 days post-injection in both eyes, and the dog was euthanized at 70 days post-injection. For the next three dogs (one injection, OS; two injections, OS; one injection, OS), cells were not visible at approximately one week post-injection (7, 8, 7 days) and were not visible for the remainder of their inclusion in the study: 33 and 63 days for dog 3, euthanized at 68 days post-injection; 28 days post-injection and 5 days post-second injection, dog 4 was adopted out; 56 days post-injection, dog 5 was adopted out. The sixth dog received injections in both eyes, with a 90-minute delay following collection prior to injection for OS while OD cells were injected immediately following collection. Cells were visible at 28 and 45 days post-injection in OS, but not in OD, and the dog was euthanized at 52 days post-injection.

**Figure 2-4:** Autofluorescence (A) and OCT (B) imaging of rAAV2-CMV-GFP transduced autologous cMSCs injected into the vitreous of a dog *in vivo* (color added in Photoshop). Thick arrow: shadow created by cell clump; thin arrow,
originally thought to be individual clusters of MSCs, but subsequently identified as an artifact of the imaging process.

2.3.5. Behavior of cMSCs *In Vivo*

As seen with OCT and ophthalmoscopic imaging (Figures 2-4 and 2-5), transduced MSCs were localizable *in vivo* immediately following injection. In dogs 3, 4, 5, and 6 OD, cells were no longer visible via OCT imaging as soon as 1 week post-injection, while in dogs 1, 2, and 6 OS, cells were visible for the duration of the study period through euthanasia. In none of the eyes did the numbers of cells that could be visualized appear to increase. This suggests that after implantation the cells ceased to proliferate. Instead, cell clumps that were visible throughout the study duration were seen to reduce in size over time, but did remain in sufficient quantities such as to be visible. Furthermore, at no time were any cells seen to invade any portion of the retina. In the 3 of the 4 dogs in which cells were not visible except immediately post-injection, the cells were seen to diffuse rapidly away from the syringe tip rather than stay in a localized clump. In the remaining dog (6 OD), cells were visible immediately post-injection, but had diffused after one week (Figure 2-5). Via autofluorescence imaging (Figure 2-4A), continued expression of the *GFP* transgene was confirmed *in vivo* and the cells were seen to maintain expression for the duration of the studies (52, 70, and 50 days).

Following the immediate diffusion of cells post-injection in dogs 3, 4, and 5, the injection protocol was modified for dog 6 OS to include a waiting period between the time of cell collection and injection, 90 minutes at 37°C and 5%
CO\textsubscript{2}, while 6 OD would receive the injection immediately following collection and transport to the surgical suite. The rationale behind this modification was the hypothesis that the cells were not able to regenerate the cell surface proteins required for adhesion that were cleaved via trypsinization during collection in the limited time prior to injection. The goal of preventing the dispersal of the injected cells was to enable their visualization \textit{in vivo} for long periods of time.

Subsequently, cells were localizable in 6 OS throughout the course of the study, while those in 6 OD dispersed post-injection (Figure 2-5B).

![Figure 2-5: Ophthalmoscopic imaging immediately following stem cell implantation (A) and one week post-implantation (B). Arrow: cluster of stem cells present immediately post-implantation. Cluster has dispersed after one week \textit{in vivo}.](image)

### 2.3.6. Microscopy

Following euthanasia, segments of glutaraldehyde-fixed retinas were embedded in epoxy resin, stained with toluidine blue, and the sections were examined with transmitted light microscopy (Figure 2-6). In accordance with the observed behavior of the transduced MSCs \textit{in vivo}, the injected cells were seen
to have not invaded the retina or disorganize the retinal architecture in any observable fashion. Compared against a retinal cross-section from an untreated TPP1<sup>+/+</sup> control dog (Figure 2-6A), the retina of an affected TPP1<sup>-/-</sup> dog that received the mock treatment (<i>GFP</i> expressing MSCs) looked appreciably normal in terms of gross morphology (Figure 2-6B). Cell loss and cell layer disorganization is apparent in the mock treated dog, but this can be attributed to CLN2-related retinal degeneration and is not a side effect of the presence of the transduced cells.

![Unaffected](TPP1<sup>+</sup>) ![Affected, Mock Treated (<i>GFP</i> MSCs)](TPP1<sup>-/-</sup>)

**Figure 2-6:** Cross-sections of retina from a TPP1<sup>+/+</sup> control dog (right) and a TPP1<sup>-/-</sup> mock treated dog (received <i>GFP</i> expressing MSCs). No disruption of the retinal morphology of the mock treated dog is visible in response to the presence of the MSCs.

Clusters of <i>GFP</i> expressing cells in the vitreous large enough to visualize using the fluorescence stereoscope were dissected from the eyecups along with the surrounding vitreous and transferred to vials containing cacodylate buffer and were then photographed with the fluorescence stereoscope (Figures 2-7 and 2-8A and B). The clumps of cells visible via OCT and opthalmoscopic imaging
while *in vivo* were localized post-euthanasia and continued *GFP* transgene expression was confirmed *in vitro*. Both singular larger clumps and multiple smaller cell groupings were recovered. A clump of autofluorescent cells that were visible pre-euthanasia in one of the treated eyes was collected postmortem, fixed, embedded in epoxy resin, and sectioned. The sections were then stained with toluidine blue (Figure 2-8C). Analysis of the cell aggregate showed that the cells formed and where suspended in an extracellular matrix post-injection *in vivo* and displayed fibroblast cell-like morphologies.

![Figure 2-7: Fluorescence micrographs taken with a fluorescence stereoscope; dogs were euthanized nine weeks post implantation of *GFP*-expressing cells. The eyes were fixed, and the corneas, irises and lenses were removed. *GFP*-expressing cells were dissected out from the vitreous of one eye; both singular large clump of cells (A and B) and smaller diffuse cell groupings (C) visible.](image)
Figure 2-8: Images of GFP-expressing MSCs in the vitreous of a TPP1⁻/⁻ dog at 52 days post-injection. The dog was euthanized and after removal of the cornea, iris and lens, the vitreous was examined for GFP fluorescence using a Leica MZFLIII stereoscope. One large aggregate of GFP-expressing cells was observed (A) as well as numerous small aggregates of individual cells which were scattered throughout the vitreous (B). The large aggregate was dissected from the vitreous, post-fixed with osmium tetroxide and embedded in epoxy resin. Toluidine blue stained sections of the aggregate revealed that it consisted of cells with fibroblast-like morphologies distributed throughout an extracellular matrix (C).

2.4. Discussion

These initial canine studies suggest that the intravitreal implantation of transduced MSCs should be safe as a long-term intraocular delivery method for therapeutic agents in the treatment of retinal degeneration. As a result of these studies, it should be feasible to transduce autologous MSCs with a gene therapy vector directing the constitutively active expression of a soluble protein that is deficient within the cells of the retina, inject the cells intravitreally, and expect that
they will survive indefinitely, will not proliferate or damage the retina or other components of the eye, and will produce and export the protein (Figure 2-9).

Two AAV2 vectors were used in this study to drive constitutive expression of the GFP reporter transgene: AAV2-CMV-GFP and AAV2-CAG-GFP. Initial studies (dogs 1-3) were conducted exclusively using the CMV version of the construct; however, in response to concerns that the promoter could eventually be turned off in vivo, thereby silencing transgene expression, later studies (dogs 4-6) utilized the CAG promoter. Similar levels of GFP expression were observed in vitro and both promoters continued to drive expression in the cells throughout the course of the studies, both in vitro and in vivo. The CAG vector did result in earlier observable GFP expression than the CMV vector, generally on the order of 2-3 days earlier. All things equal, a shorter turnaround between date of aspiration and injection is desirable, as any potential benefit of the ex vivo gene therapy approach is dependent on time of treatment relative to symptom severity and onset. While a 2-3 day difference would not likely make an appreciable difference in the long term as the general turnaround time is between 3-4 weeks, there is no reason not to employ the vector that results in faster expression.

The bone marrow derived canine MSCs were able to proliferate in vitro, reach confluency, and be passaged multiple times given sufficient time and culture resources; cells were initially followed through passage 6 while those to be injected were passaged until they reached passage 3 or 4. Once injected intravitreally, the cells were not seen to proliferate, and in at least one case the clumps of cells diminished in size over time. Whether this diminishment is the
result of cells dying off over time *in vivo* or simply detaching off of the greater clump and diffusing away through the vitreous is not known at this time. The formation of extracellular matrix holding the cell clumps together *in vivo* as seen during microscopic analysis post-euthanasia would suggest that it is unlikely the cells would simply detach and float off from the singular clump, but conversely, the ability to dissect out an intact clump of cells post-euthanasia is in and of itself sufficient evidence to demonstrate that the cells are able to survive long-term (at least 70 days post-injection) within the vitreal environment. Additionally, it is possible, as extracellular matrix was only observed between the cells that remained clumped together through euthanasia and that were capable of being dissected out, that the cells tended to diffuse throughout the vitreous unless they produced an extracellular matrix to hold them together. The balance between the rate of migration of the cells away from the injection site relative to the rate of extracellular matrix production could explain the presence or absence of sustained clumping between the dogs. Alternatively, it may be possible that the cells and the extracellular matrix holding the clumps together *in vivo* are particularly delicate and may not be able to withstand the various forces exerted by the repeated movement of the eye and vitreous over the course of multiple months. Ultimately, the exact reason for the observed decrease in size of the cell clumps over time *in vivo* is not known at this time and remains one of the biggest concerns with regards to the longevity of the proposed *ex vivo* gene therapy delivery method.
As observed, the MSCs did not proliferate within the vitreous at any time post-injection. This suggests that the vitreous either lacks the various growth factors necessary to facilitate the indefinite growth of the MSCs, as seen in vitro, or contains factors that actively inhibit proliferation. The vitreous, however, cannot be a completely inhospitable environment for the MSCs, because again, the cells were able to survive in some capacity through the end of the study and at time of euthanasia, between 50 and 70 days post-injection (Meyer et al., 2006; Tracy et al., 2015). This then suggests that the cells were able to receive sufficient oxygen and nutrients from the retina such to ensure their long-term survival, but perhaps did not receive the factors necessary to promote proliferation. As to why the cells did not undergo apoptosis in the absence of proliferative growth factors, it may be possible that the retina also provided sufficient trophic factors to the cells to prevent cell death, but insufficient quantities to either instigate proliferation or differentiation of the MSCs. All of this presupposes that it is proximity to the retina that ensures the long-term survival of the MSCs in vivo; however, this close proximity might also not be completely necessary as cells implanted into the vitreous of a dog further away from the retina were still present multiple months post-implantation. Furthermore, in the dogs in which the cells remained clumped together through time of euthanasia, the injected cells were not seen to migrate towards the retina. In the dogs in which the cells eventually dispersed over time and were no longer able to be visualized, no disruption of the retina was observed via OCT imaging. The MSCs employed in these studies may respond differently to trophic factors released by
the retina as compared to some other potential donor cell type, such as neural precursor cells, again such that prolonged cell homeostasis is achieved and no further, and in this utilization, detrimental cell processes are not triggered (Meyer et al., 2006; Tracy et al., 2015).

These studies indicate that the intravitreal implantation of genetically modified autologous MSCs represent a feasible avenue for the sustained delivery of therapeutic agents to the retina. The studies establish that the foundation for the putative ex vivo gene therapy approach is safe, i.e. that GFP-transduced autologous MSCs injected intravitreally do not appear to trigger an immune response and do not harm the retina due to their presence, and lay the groundwork for further studies investigating the approach’s efficacy in the production of therapeutic proteins in vivo in the treatment of retinal degenerative disorders.
**Figure 2-9:** Hypothesized method by which an AAV2-TPP1 transduced donor cell may produce the precursor form of TPP1 and export it extracellularly where it will be taken up by a recipient cell, transported into the lysosome, and become active.
CHAPTER 3

EX VIVO GENE THERAPY FOR PRESERVATION OF THE RETINA IN A CANINE MODEL OF CLN2

3.1. Introduction

Soluble lysosomal enzymes, including TPP1, are trafficked both inter- and intracellularly via the endosomal system (Figure 3-1). Following ligand-receptor mediated endocytosis at the cell surface, these enzymes are transported to the lysosomes where they become active (Dahms et al., 1989; Guhaniyogi et al., 2009). Therefore, supplying exogenous TPP1 to the surface of cells that lack endogenous enzyme, as in CLN2, should then be able to rectify this deficiency and restore normal molecular function. This therapeutic approach has already been previously confirmed in other lysosomal storage disorders through the systemic administration of the appropriate exogenous enzymes via the bloodstream (Chen et al, 2009; Grubb et al., 2010).

We hypothesize that if the TPP1 pro-enzyme can be continuously supplied to the ocular vitreous, it will be taken up by cells throughout the various retinal layers, localize to the lysosomes within the cells and become active, thereby ameliorating the deficiency of this enzyme in CLN2 disease. Previous and current studies have already shown that delivery, uptake, and resulting wholesale distribution of the enzyme occurs in the brain and can be accomplished via catheter-mediated TPP1 enzyme replacement as well as direct gene therapy utilizing an AAV2-CAG-TPP1 vector. The relatively short half-life of the enzyme once it has been endocytosed requires that the infusions used in the enzyme
replacement therapy approach be repeated every two weeks following initial administration (Chang et al., 2008). Such a frequent treatment course is undesirable in addressing CLN2-related retinal degeneration.

Figure 3-1: Cartoon demonstrating uptake, transport, and activation of exogenous pro-TPP1 by the cell via the endosomal network.

In preliminary studies, repeated injections of recombinant TPP1 into the vitreous of TPP1-/- dogs has resulted in significant complications including intraocular inflammation, glaucoma, and corneal swelling. Any surgery or retinal injection carries inherent risks, and while the possibility of any side effects or tissue damage as a result of a single intraocular injection may be low, for the enzyme replacement therapy approach this risk is compounded exponentially due to the requirement that treatment be continued indefinitely as long as the patient is alive. Thus, TPP1 enzyme replacement therapy does not appear to be a viable approach for preventing blindness in CLN2 disease. Direct TPP1 gene
therapy to the CNS via administration of the AAV2-CAG-TPP1 vector to the CSF, although it had a pronounced therapeutic benefit, was accompanied by chronic CNS inflammation. The possibility of a similar complication is of concern in attempting to treat the retina with injection of the gene therapy vector directly into the ocular vitreous. In addition to the risk of chronic inflammation additional limitations are associated with the use of a direct AAV2 delivery regimen, including the possibility of an immune response against the vector and the inability for repeated vector administrations due to the development of antibodies to the AAV2 proteins.

To avoid the putative risks and limitations associated with these two methods, we endeavored to deliver TPP1 to the retina via production of the pro-enzyme from transgenic cells that will reside in the ocular vitreous. We hypothesized that transgenic autologous MSCs that overexpress TPP1, when injected into the vitreous of dogs with CLN2, would supply functional TPP1 pro-enzyme to the retina via the mechanism depicted in Figure 2-9 and that delivery of the enzyme to the retina via this route will also delay and/or prevent the development and progression of the retinal degeneration and associated symptoms characteristic of dogs with CLN2 disease as has been previously established (Figure 3-2).
2.1 Enrich for mesenchymal stem cells (MSCs) in culture (passage 3; ~10-12 days).
2.2 Transduce MSCs with AAV2-TPP1 vector and allow for transgene overexpression (~3 days).

Figure 3-2: Cartoon representing the three primary steps in the therapeutic pipeline utilized in these studies. Cells are harvested from the bone marrow (1), modified in vitro to produce TPP1 (2), and injected intravitreally (3). Modified figure from Dr. Rebecca Whiting.

3.2. Materials and Methods

3.2.1. Animals

Studies employed affected purpose-bred long-haired miniature Dachshunds (n=6) that were homozygous for a one nucleotide deletion (c.325delC) in exon 4 of the TPP1 gene (TPP1-/-) and were genotyped within several weeks of birth at the TPP1 locus using an allelic discrimination assay that distinguishes between the normal and mutant alleles (Awano et al., 2006). Dogs homozygous for the deletion present with a disease course and symptoms very
similar to those of CLN2 disease in humans. Dogs were housed and bred as described in Chapter 2.2.1.

3.2.2. Ophthalmic Examinations

Prior to their inclusion in the study, all dogs received a complete ophthalmic examination at 10-12 weeks of age, before the onset of any signs of retinal or neurological impairment due to CLN2 in the affected dogs; dogs with evidence of compromised vision or any ophthalmic abnormalities deemed threatening to vision were excluded from the studies. Examinations were conducted as described in Chapter 2.2.2.

3.2.3. Bone Marrow Isolation

Bone marrow aspirates were collected from each dog at approximately 2.5-3.5 months of age utilizing the procedure described in Chapter 2.2.3.

3.2.4. Cell Culture

The bone marrow preparation was plated and the cells were maintained using the same protocol described in Chapter 2.2.4.

3.2.5. Transduction of MSCs

After reaching either passage 3 or 4, the cells were either transduced directly while attached to plates (n=3, later dogs) or harvested from the plates as described in Section 3.2.4 and transduced in suspension (n=3, earlier dogs).
The concentration of cells on each plate was estimated via density (numbers of cells per 1 mm\(^2\) then extrapolated to available surface area on plate). If transduced in suspension, the cells were pelleted after trypsinization and resuspended in SCCM prior to treatment with the transduction vector. The resulting cell suspensions were then transduced with an AAV2-CAG vector backbone containing a GFP-expression construct (SignaGen cat. No. SL00816) or a TPP1-expression construct derived from the human TPP1 cDNA (SignaGen cat. No. SL100801). Human cDNA was used in the creation of the TPP1-expression construct because good therapeutic efficacy was observed previously in enzyme replacement studies conducted by the lab utilizing human recombinant TPP1 (Katz et al., 2014). The frozen vector was thawed and an aliquot was added to the cell suspension to achieve a multiplicity of infection (MOI) of 50,000 for AAV2-CAG-TPP1 or 10,000 for AAV2-CAG-GFP and the suspension was then gently mixed. The suspension was then plated and incubated for approximately 96 hours at 37°C in 5% CO\(_2\). If transduced while plated, plates were washed with PBS and then the vector was added to a volume of SCCM and then added to the plates, with additional media added to reach either a total of 10 mL (for 100 mm • 20 mm plates) or 25 mL (for 150 mm • 25 mm plates). Regardless of transduction method, after 96 hours the medium containing the vectors was the drawn off and replaced with fresh SCCM and the cells were cultured in this medium until used for implantation.
3.2.6. *In Vitro* Examination

AAV2-GFP transduced cells were evaluated for GFP expression as described in Chapter 2.2.6.

3.2.7. TPP1 Enzyme Activity Assay

To assess TPP1 expression in cells transduced with the AAV2-CAG-TPP1 vector, plates of transduced and untransduced cells from the same dog were washed with PBS and SCCM was added. Twenty-four hours later, the conditioned SCCM was drawn from the culture plates and frozen at -80°C for later determination of TPP1 enzyme activity. The rate of TPP1 output per cell transduced with the AAV2-TPP1 vector was quantified by measuring TPP1 enzyme activity in the 24 hour conditioned medium (Chang et al., 2008).

Conditioned SCCM (10µL) was added to wells of a 96-well black wall plate containing 80µL sodium citrate buffer (pH 4.0). The enzyme reaction was initiated by addition of 10µL substrate (250 µM Ala-Ala-Phe 7-amido-4-methylcoumarin in sodium citrate buffer, pH 4.0) (Sigma cat. No. A3401). Plates were then incubated in a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA) at 37°C and fluorescence emission was monitored with 360 nm excitation and a 460 nm emission filter. Specific activity was used to calculate the amount of TPP1 per volume of SCCM, divided by the estimated number of cells per plate, then used to determine TPP1 output per unit of time per cell. Specific activity was determined by performing the same assay with known amounts of purified TPP1 as standards (R&D System cat. No. 2237-SE-010).
3.2.8. Intravitreal Injection of cMSCs

The *TPP1*- and *GFP*-expressing MSCs were implanted into the vitreous of the same dog from which the cells were derived; *TPP1* cells were implanted in the left eye of affected dogs (OS) (n=6), an equal number of *GFP* cells were implanted in the contralateral right eye (OD) (n=4). Injections of *GFP* cells were used as controls in 4 of 6 dogs; the remaining two dogs did not receive any treatment in the eye contralateral to the treated eye. Implantation was performed approximately 2 to 3 weeks after bone marrow aspiration, at approximately 14 weeks of age. Starting 1 week prior to the planned injection, cyclosporine administration was initiated at 25 mg BID. Cyclosporine administration continued at this dose until the dog reached a weight of 4 kg after which the dose was increased to 35 mg BID. Cyclosporine administration at this dose was continued until the dog was euthanized. On the day of injection the cells were harvested from the culture plates as described previously for cell passaging (Section 3.2.4), pelleted, and then resuspended in either MEM alpha (dogs 1-3) or SCCM (dogs 4-6) at concentrations of 9.5 (*TPP1*), 12.5 (*TPP1*), 30 (*TPP1/GFP*), 31.3 (*TPP1/GFP*), 33.3 (*TPP1/GFP*), 48 (*TPP1/GFP*) million cells per mL and either injected immediately or held at 37°C for 60 to 90 minutes. Starting 1.5 hours prior to the planned injection, a drop of prednisone acetate 1% ophthalmic suspension and a drop of topicamide 1% ophthalmic solution were administered to the corneal surface every 10 minutes for 30 minutes. Just prior to the injection procedure, 0.2 mg/kg dexamethasone (2 mg/mL) was administered
intravenously. The dogs were placed under general anesthesia as described previously for the bone marrow aspirations and maintained on a ventilator. The eyes were washed with dilute betadine. The cells in the tube were uniformly suspended by gently drawing them up into a 1 mL sterile syringe with a 1 inch 27-gauge needle and expelling them several times and the suspension was then drawn into the syringe. With or without the aid of an operating microscope and a Machemer Magnifying Vitrectomy lens (Ocular Instruments, Bellevue, WA), 200 to 400 µL of the cell suspension was injected into the vitreous with the needle entry point 7 mm posterior to the limbus and needle trajectory pointed towards the optic nerve, with the injection made from the top of the eye. Immediately following the procedure 2 mg of triamcinolone acetonide was injected sub-Tenon’s. Prednisolone acetate 1% ophthalmic suspension was administered twice daily for one week following the procedure and tropicamide 1% ophthalmic solution was administered once daily (as needed) for one week following the procedure. After one week, 2.2 mg/kg Carprofen PO BID was administered if uveitis was observed.

3.2.9. Optical Coherence Tomography

Immediately and then one week after injection, eyes were imaged with funduscopy and with a Spectralis HRA/OCT imaging system (Heidelberg Engineering, Heidelberg, Germany) as described in Chapter 2.2.8.
3.2.10. Quantification of Retinal Lesions

Wide-angle cSLO fundus images were analyzed using Adobe Photoshop Extended. Lesions were manually outlined using the lasso tool, and the “record measurements” function was used to calculate the area occupied by all lesions combined. The total area of the superior retina evaluated for lesion content, excluding the optic nerve head, was also determined using the lasso tool and “record measurements” function. The percentage of the total area of the superior retina covered by lesions was then calculated from these data.

3.2.11. Electroretinography

Bilateral electroretinogram (ERG) evaluations were performed for all treated $TPP1^\Delta$ dogs on an approximately monthly basis beginning 1 month post-injection as described previously (Whiting et al., 2013). Dogs were prepared for ERG recording in ambient room light. Prior to recording, both pupils were dilated with 1% tropicamide, and dogs were deeply sedated with intramuscular administration of dexmedetomidine (30 - 40 µg/kg). ERGs were elicited bilaterally and recorded simultaneously with a commercial instrument (HMsERG model 2000; RetVet Corp., Columbia, MO). The right and left mini-Ganzfeld domes were positioned approximately 2 cm from the corresponding eye. Each ERG session consisted of scotopic and photopic recordings in accordance with the Dog Diagnostic Protocol, recommended by the European College of Veterinary Ophthalmology, primarily for evaluation of rod and cone function (Ekesten et al., 2013). During a 20 minute period of dark adaptation, scotopic
low-intensity rod responses were elicited at a stimulus intensity of 10.2 log photons/cm²/s (0.01 cd.s/m²) with 4 minutes of dark adaptation between recordings. Following this, scotopic responses were elicited using flashes of 12.65 and 13.2 log photons/cm²/s (3 cd.s/m² and 10 cd.s/m²) to evaluate mixed rod and cone function. The eyes were then exposed to diffuse white light at a luminance of 13.65 log photons/cm²/s (30 cd/m²) for 10 minutes, immediately after which responses to single 12.65 log photons/cm²/s (3 cd.s/m²) flash stimuli were recorded. This was immediately followed by evaluation of responses to 30-Hz photopic flicker stimuli at the same light intensity. ERG waveforms in all recordings were evaluated, and the amplitudes and implicit times for the a- and b-waves were measured as previously described (Whiting et al., 2013).

3.2.12. Euthanasia and Necropsy

End stage dogs (approximately 11 months of age) were sedated with dexmedetomidine and euthanized with Fatal Plus (sodium pentobarbital). Tissues were collected and fixed for microscopy as described in Chapter 2.2.9.

3.2.13. Light Microscopy

Segments of glutaraldehyde-fixed retinas were prepared for light microscopy and examined as described in Chapter 2.2.10.
3.3. Results

3.3.1. Cell Culture and Transduction of cMSCs

Cells from bone marrow aspirates were allowed to attach to 100 mm \( \times \) 20 mm plates for a period of 48 hours, during which the plates were washed repeatedly at 24 and 48 hours post-plating with 1X PBS to remove non-adherent cells, the majority of which were red blood cells. The plates were cleared of any remaining non-adherent cells after the 48 hour washes, and the remaining cells were identified as mesenchymal stem cells (MSCs) by their morphology and adherence to plastic (Williams and Hare 2011; Pittenger et al., 1999). Cells were passaged from the initial plating (passage 0, P0) to P1 after they reached initial confluence (here described as the formation of symmetric colonies spanning the entirety of the available growth surface), 2-6 days in SCCM media. During the P0-P1 passage, the initial 100 mm \( \times \) 20 mm plates were expanded to 150 mm \( \times \) 25 mm plates at a 1:1 expansion and were maintained on these plates through subsequent passages at a 1:2 expansion. Time between subsequent passages (P2 and P3) varied between dogs, with some cell populations reaching P2 and P3 confluency after 24-48 hours following the previous passage, while others grew more slowly between passages and required 3-5 days to reach confluency. Regardless of rate of growth, all cells displayed typical MSC morphology and cell morphology remained indicative of an MSC lineage through repeated passage (Pittenger et al., 1999).
Initial GFP expression was detectable 96 hours post-transduction and increased in intensity over time, reaching a stable high level of intensity approximately 5 days after transduction. GFP expression remained stable for at least two passages post-transduction. A high percentage of observable cells (near 100%) displayed some level of GFP fluorescence in vitro, with similar transduction efficiency observed at 10,000 MOI and 50,000 MOI (Figure 2-2).

Cells were transduced with the rAAV2-CAG-TPP1 vector at 50,000 MOI (Figure 3-3). TPP1 activity within the conditioned media was confirmed and the estimated release of enzyme by the MSCs in vitro was approximately 3 to 5 pg per cell per 24 h.

Figure 3-3: Recombinant adeno-associated vector, serotype 2, backbone utilized in the transduction of cMSCs. Vectors consisted of flanking long terminal repeats (LTRs), a promoter (CAG), multiple cloning site containing either GFP or hTPP1 cDNA, and a polyA tail. The AAV2-GFP vector was used to transduce cells to be used for control; the AAV2-TPP1 vector was used to transduce cells for treatment.

3.3.2. OCT and Ophthalmic Exams

For each of the dogs included in the study, AAV2-CAG-GFP or AAV2-CAG-TPP1 transduced MSCs were injected into the right eye (OD) as controls or into the left eye (OS) as treatment, except for one dog which received a mixture of GFP and TPP1 cells into one eye (OS) and a second which received only
TPP1 cells (OS). Optical coherence tomography (OCT) imaging was used to locate or attempt to locate the cells at various time points post-injection as to monitor the survival of the cells in vivo and to ascertain whether the cells were migrating or expanding. In all cases, cells were visible immediately following injection; however, the visibility of the cells via OCT was variable among the dogs at future time points. For the first dog examined (mixture of cells, OS), cells were visible via ophthalmic exam 1, 2, 7, and 28 days post-injection and were localizable via OCT at day 28 as a small, diffuse clump of cells within the dorsal vitreous. Cells were no longer visible through ophthalmic exam or OCT beyond this time. Grade 1 retinopathy was diagnosed at 71 days post-injection in both eyes and remained at grade 1 through euthanasia at 192 days post-injection.

The second dog received one injection (TPP1, OS) into the dorsal vitreous; cells were not visible on ophthalmic exam at 1 day post-injection and were unable to be visualized by OCT at 6 days. Cells were not visible during any subsequent exams or OCT. Grade 1 retinopathy was confirmed via OCT at 114 days post-injection and was seen to be more severe in OD relative to OS. Retinopathy progressed to grade 2 in OD by 234 days while remaining grade 1 in OS. By 261 days, both eyes had progressed to grade 2, and the dog was euthanized at day 267. The third dog received OS and OD injections; cells were visible 1 day post-injection on exam in both eyes but by 5 days were not visible on OCT. Cells were localized in both eyes on exam at 48 days post-injection; however, they were not visible on subsequent exams or OCT. Retinopathy grade 1 was diagnosed in both eyes at 131 days and was sustained through euthanasia at
239 days post-injection. The fourth dog received OS and OD injections, with the GFP fluorescence easily visible in OD immediately post-injection. No cells were visible at 8 days post-injection via OCT imaging and were not visible again subsequently. Retinopathy grade 1 was diagnosed at 31 days in OD, while OS remained grade 0. Retinopathy progressed to grade 2 by 133 days in OD; OS remained grade 0. By 220 days, OD had progressed to grade 3 while OS remained grade 0, and the dog was euthanized. The fifth dog received OS and OD injections, with cells visible on exam and OCT 1 day post-injection. Cells were visible at 6 days on exam and 7 days with OCT but at no other recorded time points. Retinopathy grade 1 was present in OD by 28 days and remained constant through time of euthanasia at 168 days; OS remained at grade 0 through the course of study. The sixth dog received OS and OD injections with barely visible cell clumps visible 1 day post-injection. A small cell clump was seen at 5 days in OS on exam, but was not visible at 6 days with OCT. Grade 1 retinopathy was confirmed at 61 days in OD, with OS at grade 0. By 88 days, OD progressed to grade 2 retinopathy and OS had developed grade 1. These grades were maintained through euthanasia prior to disease end-stage at 210 days post-injection. A seventh dog received both OS and OD injections, but developed a complete retinal detachment in OS apparently unrelated to the injection and was subsequently un-enrolled from the therapy trials.
3.3.3. ERG

Electroretinographic (ERG) responses were recorded from dogs 2 through 6 at approximately 4 months of age and then again every month until reaching 10-11 months of age, roughly disease end-stage. Scotopic high-intensity responses were elicited using flashes of 13.2 log photons/cm²/s (10 cd.s/m²) to evaluate mixed rod and cone function and to gauge the relative health of the retinal layers (Figures 3-4, 3-5, 3-6).

![ERG Graph](image)

**Figure 3-4:** Stereotypic high-intensity scotopic ERG responses from 6-month old normal TPP1+/+ and CLN2-affected TPP1−/− long-haired miniature Dachshunds utilized in these studies. Affected dogs have a reduced a-wave and a greatly diminished b-wave that both progressively deteriorate. Figure from Dr. Rebecca Whiting.

The standard ERG series was conducted for each dog at each time point (4 through 10/11 months of age) in order to elicit a rod-system response, combined rod and cone system responses to a strong flash, a single-flash cone response, and a cone-system response (Figure 3-7).
The ERG responses from each protocol were averaged between the five dogs in the study and compared quantitatively across treatment groups (normal, affected untreated, and affected treated). The normal group was composed of normal, untreated TPP1+/+ dogs that received no treatment, while the affected groups consist of the responses elicited from the OS (received the ex vivo gene therapy treatment) and OD (received the control treatment) from each of the five affected TPP1-/- dogs enrolled in the study (dogs 2 through 6). Average a-wave amplitude plots for high-intensity rods and cones and cones (Figure 3-8 A and B), average b-wave amplitude plots for rods, high-intensity rods and cones, cones, and flicker (Figures 3-8 C, D, E, and F) and the b:a wave amplitude ratio were generated (Figure 3-8 G).
Figure 3-5: Cross section of the retina from a normal TPP1*/* long-haired miniature Dachshund demonstrating the multiple cell layers that make up the canine retina (A). A high-intensity scotopic ERG response consists of two waves, the a-wave and the b-wave. The a-wave is generated by the rod and cone cells within the photoreceptor layers while the b-wave is derived from cells in the inner retina (plexiform and nuclear layers). Modified figure from Drs. Martin Katz and Rebecca Whiting.
Figure 3-6: Scotopic high-intensity responses generated using flashes of 13.2 log photons/cm²/s (10 cd.s/m²) from CLN2-affected TPP1-/- long-haired miniature Dachshunds. Dogs received variable quantities of TPP1 transduced MSCs (OS), with GFP transduced cells used as control (OD).
Figure 3-7: Representative ERG tracings from an affected dog at multiple time points post injection at 3.3 months of *TPP1* (red traces, approx. 10 million) and *GFP* (blue traces, approx. 10 million) transduced cells. Scotopic rod recordings (A), 10 cd s/m² mixed rod and cone recordings (B), photopic cone single flash (C), and 30 Hz flicker recordings (D).
Figure 3-8: Averaged scotopic high-intensity rods and cones (10 cd.s/m²) a-wave amplitudes from normal untreated TPP1⁺/⁺ dogs (black), affected untreated TPP1⁻/⁻ eyes (red), and affected treated TPP1⁻/⁻ eyes (purple) (A). Averaged photopic cones (3 cd.s/m²) a-wave amplitudes from same treatment groups (B).
Figure 3-8 (cont.): Averaged scotopic rods (0.01 cd.s/m²) b-wave amplitudes from normal untreated TPP1<sup>+/+</sup> dogs (black), affected untreated TPP1<sup>−/−</sup> eyes (red), and affected treated TPP1<sup>−/−</sup> eyes (purple) (C). Averaged scotopic high-intensity rods and cones (10 cd.s/m²) b-wave amplitudes from same treatment groups (D).
Figure 3-8 (cont.): Averaged photopic cones (3 cd.s/m²) b-wave amplitudes from normal untreated TPP1+/+ dogs (black), affected untreated TPP1−/− eyes (red), and affected treated TPP1−/− eyes (purple) (E). Averaged 30-Hz photopic flicker b-wave amplitudes from same treatment groups (F).
Figure 3-8 (cont.): Ratio of b-wave to a-wave amplitudes of scotopic high-intensity rods and cones (10 cd.s/m²) responses from normal untreated TPP1<sup>+/+</sup> dogs (black), affected untreated TPP1<sup>-/-</sup> eyes (red), and affected treated TPP1<sup>-/-</sup> eyes (purple) (G).

3.3.4. Retinal Lesions

Affected TPP1<sup>-/-</sup> dogs developed retinopathy, raised lesions forming on the retina (Whiting et al., 2015), and were evaluated on a monthly basis beginning at 4 months of age for the progressive development of detachments and changes in retinopathy grade (Figure 3-9): grade 1, less than 15% of the retina affected by lesions; grade 2, greater than 15% but less than 30%; grade 3, 30% or greater of the retina affected. For two of the six dogs in the study (dogs 1 and 3), both the eye (OS) that received the TPP1 transduced cells and the eye (OD) that received GFP transduced cells were classified as grade 1. Both of dog 2’s eyes eventually progressed to grade 2, but the progression was much slower in OS than OD (Figure 3-10). The treated eye of dog 4 remained at grade 0 (no lesions visible) throughout the entire course of study, while the control eye eventually reached grade 3 by end-stage. Dog 5’s treated eye remained at grade 0 while
the control eye was grade 1, and in dog 6 the treated eye reached grade 1 while the control eye progressed to grade 2 (Figure 3-11).

Figure 3-9: CLN2-affected TPP1\textsuperscript{-/-} long-haired miniature Dachshunds utilized in this study develop progressive focal detachment lesions on their retinas. Normal TPP1\textsuperscript{+/-} dogs do not develop lesions as compared with an affected dog (A and B). Fundus images were analyzed and used to calculate the area occupied by all lesions combined and relative to the total area of the superior retina (C). OCT cross-section of a retinal detachment from an affected, demonstrating the retina detaching from the back of the eye.

For each of the six dogs, the development of the focal detachment lesions was more pronounced in the control eye than in the treated eye, even for those dogs that reached equivalent retinopathy grades in both eyes. Lesions were seen to develop more quickly and were larger in size, number, and general severity in the control eyes relative to the treated eyes. Furthermore, the development and progression in severity of lesions in at least one dog was seen
to suggestively correspond with diminishment of the scotopic high-intensity rods and cones ERG response (Figure 3-12).

**Figure 3-10:** Lesion progression was delayed in treated eyes relative to untreated control eyes. Pictured: dog 2. Figure from Dr. Rebecca Whiting and Lauren Gillespie.
Figure 3-11: Severity of lesions and retinopathy grade at CLN2 disease end-stage was variable between dogs. Left: dog 4; middle: dog 5; right: dog 6.
Figure 3-12: Scotopic high-intensity rods and cones ERG responses from dog 4, which received approximately 10 million *TPP1* transduced MSCs (OS, red) and approximately 10 million *GFP* transduced MSCs (OD, blue) with corresponding fundus images of the OS and OD. Percent lesion area represents the area of all lesions combined as a percentage of the total area of the superior retina.
3.3.5. Histopathology

Following euthanasia, segments of glutaraldehyde-fixed retinas from dog 1 were embedded in epoxy resin, stained with toluidine blue, and the sections were examined with transmitted light microscopy. The dog’s OS received treatment, consisting of roughly 1.9 million AAV2-TPP1 transduced cMSCs and 600K AAV2-GFP transduced cMSCs, for a total of 2.5 million cells. The cells were injected at age 15 weeks and the dog was euthanized at 42 weeks. OD did not receive treatment and was used as a control (Figure 3-13). The retina from the untreated eye showed increased cell loss within the outer and inner nuclear layers and thinning of the outer plexiform layer, as compared to the retina from the treated eye.

Figure 3-13: Retinal cross sections from a CLN2-affected TPP1-/- long-haired miniature Dachshund that received approximately 1.9 million TPP1 expressing MSCs (left) in OS while the OD remained untreated as a control (right). The red box highlights the cell dropout in the outer nuclear layer in the untreated retina compared to the treated.
3.4. Discussion

In this study we have documented for the first time an effective therapy for the treatment and delay of CLN2-related retinal degeneration in a TPP1<sup>−/−</sup> long-haired miniature Dachshund model. These canine studies suggest that the intravitreal implantation of transduced MSCs should be safe as a long-term intraocular delivery method for TPP1 in the treatment of CLN2-related retinal degeneration. As a result of these studies, it should be feasible that the <i>ex vivo</i> gene therapy approach should be translatable to humans: transduce autologous MSCs with a gene therapy vector directing the constitutively active expression of <i>TPP1</i>, inject the cells intravitreally, and expect that they will survive indefinitely, will not proliferate or damage the retina or other components of the eye, and will produce and export the enzyme to be taken up by the retina and prevent its degeneration.

The clinical benefits of the <i>ex vivo</i> gene therapy approach appeared to occur in a dose-dependent fashion, though insufficient numbers of dogs were employed in the studies in order to be able to determine this conclusively. Dogs 1 and 2 received approximately 1.9 and 2.5 million <i>TPP1</i> expressing MSCs relative to the approximately 9 to 12.5 million cells received by dogs 3-6, a 4.9x increase on average in the number of cells injected. The progression of disease-related retinal degeneration was not completely ablated in any of the dogs, including those which received the highest number of cells, but the increased delay in progression observed in the dogs receiving the higher doses suggests that with a sufficiently large enough dose it may be possible to delay
degeneration indefinitely. The results from the dog shown on Figure 3-12 are particularly promising in that the treatment completely prevented the development of retinal detachment lesions and almost completely preserved the ERG responses. If we are able to consistently replicate this result in subsequent dogs, this method of treatment should be ready for testing in children with CLN2 disease.

To consistently obtain the best result observed may require higher doses of TPP1-expressing MSCs. Achieving this larger dose may, however, be difficult as due to increased intraocular pressure generated by the increase in vitreal volume as a direct result of the cell injections, there is a limit to the volume of cells that can be injected at any one time. Within these studies, approximately 12 million cells suspended in 0.25-0.3 mL of media was deemed the largest safe single dose that could be administered without deleterious effect. Any injected volume higher than this necessitated the removal of vitreous humour due to increased intraocular pressure. While there appears to be a hard limit to the number of cells and volume that can be injected at any one time, it should be possible to inject what would constitute a larger dose by employing repeated injections spaced sufficiently far apart in time so as to allow any increase in intraocular pressure generated by one injection to subside before a second injection. In this fashion it could be possible to double or triple the total number of injected cells by splitting them over a series of injections. One potential limitation with this method, however, is that it is unclear if these repeated injections would achieve the same protective effect as seen by the single dose in
these studies due to the necessary delay between injections. The presence of the initial quantity of cells could be sufficient to delay degeneration long enough, and the results from this study would appear to bear this out, that subsequent injections could successfully achieve an additive effect and prevent degeneration completely. Alternatively, an enhanced therapeutic benefit may be possible by increasing the level of TPP1 expression per MSC injected by modifying the transduction process either by using a higher MOI of the vector or using a promoter that would increase TPP1 production.

The gradual diminishment in photoreceptor function reflected in the ERG data makes sense when in context with regards to the location of the MSC cells within the vitreous. As mentioned previously (Figure 3-5 B), the a-wave is generated by the rod and cone cells within the photoreceptor layers, which are located the most posteriorly of all the retinal layers from the vitreous. As the cells that produce the TPP1 enzyme are located within the vitreous, the enzyme must perfuse through the rest of the retina before reaching the photoreceptors. Simply, the rod and cone cells are the last cells to have access to the exogenous TPP1. The orientation of the retinal layers relative to the vitreous suggests that the TPP1 may be fully preserving the cells within the inner retina, including those cells that contribute to the b-wave. However, because the amplitude of the b-wave is directly proportional to the amplitude of the a-wave, i.e. the inner retina can only elicit a concordant response dependent on the intensity of the stimulus it receives from the photoreceptors, a preserved and normally functioning inner retina would still elicit subnormal b-wave amplitudes if the photoreceptors were
still degenerating regardless. This is best observed in the b:a wave amplitude ratio for dogs 2-5; the ratio for the treated eyes is almost normal, while the untreated eyes show clear degeneration (Figure 3-8 G). The near-normal b:a wave ratio of the treated eyes indicates that the inner retina is functioning relatively normally in proportion to the stimulus it is receiving from the photoreceptors. Most interestingly, these data taken together suggest that the limitations of the treatment reflected in the ERG data at end-stage may instead be due to the inability of the TPP1 enzyme to perfuse throughout the entire retina to prevent a later stage effect of the disease, rather than a waning effect or reduced production of the enzyme over time. Commercially available anti-TPP1 antibodies are currently being tested with immunohistochemistry in order to localize TPP1 within the retina of the affected treated dogs.

Unlike the less immediately visible preservation of the inner retina seen in the ERG results, the near total abrogation of the progressive retinopathy in the treated eyes is far more readily apparent. In 5 out of the 6 treated dogs, the treated eye either did not develop lesions (dogs 4 and 5) or if it did, only reached grade 1 retinopathy by disease end-stage. The remaining treated eye, that from dog 2, did eventually reach grade 2, but the rate of progression in doing so was much slower in comparison to the control eye. In comparison, the control eyes, either untreated or receiving GFP transduced MSCs, had reached grades 1, 2, and 3 by end-stage. Most impressively, the dog that displayed the most severe lesion progression in its control eye and ultimately reached grade 3 was also one of the two dogs that remained completely lesion free (grade 0) in the contralateral
treated eye (dog 4, Figure 3-12). This dog also had the best preservation of retinal function as measured by ERG response. While the biological explanation for the development of retinopathy in the CLN2 long-haired miniature Dachshund model is currently unknown, the presence of the retinopathy is clearly associated with the *TPP1* mutation, as only dogs homozygous for the c.325delC mutation in exon 4 of the *TPP1* gene were seen to develop retinopathy. Regardless of the genetic explanation for the formation of lesions in this model, it is clear that the presence of exogenous TPP1, as present in the ocular vitreous as a result of the *ex vivo* gene therapy approach, is able to either prevent the formation of lesions entirely, or if lesions do eventually form, delay their formation and overall progression in severity.

The ERG data suggests that the TPP1 excreted by the transduced cells located within the ocular vitreous is able to perfuse through the retina and preserve function, at least as far into the retina as the inner layers. The histopathology data supports this conclusion, as there is greater conservation of the retinal layers, up to and including the outer nuclear layer, in *ex vivo* gene therapy treated eyes relative to control (Figure 3-13). Cell dropout is more apparent in the control eye, with fewer cells visible in the inner and outer nuclear layers, as well as greater thinning of the inner plexiform layer relative to treated. It is apparent that there is at least some degree of preservation of retinal structure as a result of the treatment method.

The ERG, retinopathy, and histopathology results from this study positively indicate that the AAV2-*TPP1* transduced MSCs are able to
continuously supply the TPP1 pro-enzyme to the ocular vitreous. Based on the results seen in the treatment eyes versus the control, it is clear that the enzyme is being taken up by cells throughout most, but potentially not all, of the retinal layers, and is successfully localizing to the lysosomes within the cells and becoming active, thereby ameliorating the deficiency of this enzyme in CLN2 disease. No deleterious side effects were observed as a result of the treatment. At no point were cells observed to exert any degree of retinal toxicity, invade the retina, or cause any other form of damage or stress as a result of their being injected into the vitreous. As a result, and due to the high degree of similarity between the canine model of CLN2 disease presentation and that in humans, the ex vivo gene therapy approach should be readily applicable for the treatment of CLN2-related retinal degeneration in humans.
CHAPTER 4

EX VIVO GENE THERAPY FOR PRESERVATION OF THE CENTRAL NERVOUS SYSTEM IN A CANINE MODEL OF CLN2

3.1. Introduction

Using the canine model, we propose to evaluate an approach for the sustained delivery of the TPP1 pro-enzyme to the brain and central nervous system (CNS) using transgenic autologous MSCs expressing TPP1 injected into the cerebral spinal fluid (CSF) intracerebroventricularly (ICV) or intracisternally (IC). We hypothesize that the sustained delivery of TPP1 pro-enzyme into the CSF via an ex vivo approach will inhibit brain degeneration and preserve neurological function without the need for repeated infusions of the TPP1 pro-enzyme or the complications associated with direct gene therapy to the CNS. Based on our previous studies with enzyme replacement therapy and direct gene therapy we know that if the TPP1 pro-enzyme can be continuously supplied to the CSF, it will be taken up by cells throughout the CNS and localize to the lysosomes within the cells and become active, thereby ameliorating the deficiency of this enzyme in CLN2 disease. Previous and current studies have already shown that delivery, uptake, and resulting wholesale distribution of the enzyme occurs in the brain and can be accomplished via catheter-mediated TPP1 pro-enzyme replacement as well as direct gene therapy utilizing a AAV2-CAG-TPP1 vector (Katz et al., 2014).
With regards to infusions of recombinant TPP1 into the CSF, the relatively short half-life of the enzyme once it has been endocytosed requires that the infusions be repeated every two weeks following initial administration (Chang et al., 2008; Katz et al., 2014; Vuillemenot et al., 2015). Additionally, the catheters have been observed to become occluded over time and can be a source of significant and in some cases life-threatening inflammation. Some risks and possible limitations are also associated with the use of a direct AAV2 delivery regimen, foremost of which are the possibility of an immune response against the vector and the inability for repeated vector administrations due to the development of antibodies to the AAV2 proteins. In addition, every dog that received injections of AAV2-TPP1 into the CSF exhibited chronic brain inflammation that was only responsive to repeated steroid treatment, which entails significant side effects. To avoid the putative risks and limitations associated with these two methods, we propose to deliver TPP1 via production of the pro-enzyme from transgenic cells that will reside in the CSF. We hypothesize that transgenic autologous mesenchymal stem cells (MSCs) that overexpress TPP1, when injected into the CSF of dogs with CLN2, will supply functional TPP1 pro-enzyme to the brain and CNS and that delivery of the enzyme to the brain via this route will also delay and/or prevent the development and progression of the neuronal degeneration and associated symptoms characteristic of dogs with CLN2 as has been shown in previous enzyme replacement studies. Based on our studies with intravitreal injections, we also hypothesize that this approach will
not result in the kind of chronic brain inflammation we observed in dogs that were treated with ICV injection of AAV2-TPP1.

If the proposed therapy is successful in providing sustained levels of the TPP1 pro-enzyme in the brain and CNS to a similar degree as seen in previous CLN2 enzyme replacement and gene therapy studies, then it may serve as a template for the treatment of other CNS disorders as a means of therapeutic agent delivery. Other CNS-related lysosomal storage diseases with which systemic enzyme replacement therapy has shown some measure of success, such as α-mannosidosis, aspartylglucosaminuria, Gaucher disease type III, metachromatic leukodystrophy, and Hunter, Hurler, and the Sanfilippo syndromes (MPS IIIa-IIIId), are ideal starting points for the adaptation of the proposed therapy (van Karnebeek et al., 2012). In each of these disorders, as in CLN2, there is a deficiency of a single soluble enzyme that functions within the lysosome. It should be feasible to produce autologous transgenic MSCs that would produce the deficient enzyme in each disorder and inject them into the CSF as a permanent or at least long-lasting method of supplying the enzyme and preventing CNS symptoms. This study would lay the foundation for the treatment of these disorders by this approach as well as serve as a model for delivering other therapeutic agents to the brain and CNS for sustained periods. This approach could also be used as a means of sustained delivery of therapeutic agents to the brain in more common disorders such as Alzheimer and Parkinson’s diseases.
4.2. Materials and Methods

4.2.1. Animals

Studies employed affected purpose-bred long-haired miniature Dachshunds (n=2) that were homozygous for a one nucleotide deletion (c.325delC) in exon 4 of the TPP1 gene (TPP1 /-) and were genotyped within several weeks of birth at the TPP1 locus using an allelic discrimination assay that distinguishes between the normal and mutant alleles (Awano et al., 2006). Dogs were housed and bred as outlined in Chapter 2.2.1.

4.2.2. Neurological and Physical Examinations

Dogs received physical and neurological examinations beginning at 11-13 weeks of age, post-bone marrow aspiration. Body weight was recorded weekly. Neurological examinations were performed weekly to detect signs of neurological dysfunction and were subjectively gauged against standardized clinical neurologic examination (Lorenz et al., 2011; Sanders et al., 2011). Briefly, examination included observation and testing of: mentation, posture (involuntary movement), and gait; cranial nerves; postural reactions (proprioceptive placement, paw replacement, hopping, wheelbarrow, tactile placement, and extensor postural thrust); spinal reflexes (myotatic and flexor); and sensory testing. Gait was assessed as normal or abnormal with presence of ataxia (cerebellar, general proprioceptive, vestibular) and paresis (ambulatory, nonambulatory). Postural reactions, spinal reflexes, cranial nerve tests, and
sensation were assessed as intact, decreased, or absent. Additionally, dogs were evaluated for abnormal movement and seizures (Katz et al., 2014). Cerebral spinal fluid (CSF) was drawn at approximately 2, 5, and 6 months of age and analyzed for the presence of red blood cells, total nucleated cells, and CSF micro protein.

4.2.3. Bone Marrow Isolation, Cell Culture, and cMSC Transduction and Evaluation

Bone marrow aspirates were collected from each dog at approximately 2.5-3.5 months of age using the procedure as outlined in Chapter 3.2.3. Cells were collected from the aspirates and were cultured and transduced in vitro with a GFP- or a TPP1-expression construct as previously described in Chapter 3.2.4 and 3.2.5. Cells were monitored for expression of both GFP and TPP1 transgenes (see Chapter 3.2.6 and 3.2.7).

4.2.4. Intracerebroventricular and Intracisternal Injection of cMSCs

Intracerebroventricular (dog 1) injections were performed over 25 min using the Brainsight neuronavigation system equipped with a 26-gauge 12-cm needle. Intracisternal (dog 2) injections were performed using a 22-gauge 1.5-in needle over 10 min.
4.2.5. Magnetic Resonance Imaging

The brains of dogs included in the study were examined by magnetic resonance imaging (MRI) beginning at approximately 3 months of age, approximately 1 week prior to the intracerebroventricular (ICV) injection to locate the ventricles, and then subsequently every 6 weeks thereafter. Dogs were placed under general anesthesia for MRI acquisition. Imaging was performed with a 3-Tesla instrument (Vantage Titan 3T; Toshiba America Medical Systems, Inc., Tustin, CA). Pulse sequences were selected to obtain T2-weighted and FLAIR sequences in three planes. In addition, a 3D isotropic voxel T1W fast spoiled gradient-echo sequence was obtained for surgical planning of ICV or IC placement and volumetric studies.

MRI images were analyzed using Brainsight software (Rogue Research, Montreal, Quebec, Canada) to determine changes in brain ventricular volume as a means of assessing brain atrophy. Each MRI slice through the brain was manipulated to isolate the ventricles, after which the software automatically assembled the ventricular images from each slice into a 3D image of the ventricular system. Images were examined prior to volumetric determination and manual adjustments were made if necessary to correct for possible errors made by the automated boundary detection. Total ventricular volume was calculated by the software from the composite 3D image (Katz et al., 2014).
4.2.6. T-Maze

A T-maze apparatus and testing protocol developed by CanCog Technologies (Toronto, Ontario, Canada) with slight modifications was used to objectively test dogs included in the study for memory and reversal learning. The protocol and testing apparatus was previously described in detail, and was used in an identical fashion in these studies (Sanders et al., 2011; Katz et al., 2014). Each dog underwent an initial training period from 3 to 4 months of age in the running of the T-maze and in the execution of the testing parameters, after which they were tested on a monthly basis for their ability to achieve a consistent performance criterion in the T-maze. Briefly, the T-maze consisted of a start box, a runway, and left and right reward arms. Monthly testing consisted of three phases: preference determination, preference reinforcement, and reversal learning. Preference determination consisted of determining which side of the T-maze the dog inherently preferred, through the placement of a food reward on each reward arm of the maze and then subsequently allowing the dog to freely navigate the maze. Once preference reinforcement was completed, the dogs were transitioned to reversal learning. The food reward was placed on the side of the maze opposite the preferred/reinforced side. The dog was then allowed to navigate the maze 10 times per day, and each choice was deemed correct or incorrect. The food reward was then switched again, back to the original placement established in the preference determination phase, and the test was continued until the dog reached criterion a third time. Finally, the food reward was switched again to the opposite side and the dog was tested until it reached
criterion. Each dog was scored on the number of incorrect choices required for it to reach criterion performance in the reversal learning phase.

4.3. Results

4.3.1. Neurological and Physical Examination

The first CSF analysis, at age 2 months, from dog 1 showed 0 red blood cells (RBCs) per µL of CSF, a total nucleated cell count (TNCC) of 0 per µL CSF, and a CSF mirco protein count of 16 mg/dL CSF. The second CSF analysis, at age 5 months, showed 1 CSF RBC per µL, 2 TNCC per µL, and 17 mg/dL of CSF micro protein, while the third analysis, at 6 months, showed 634 CSF RBCs per µL (a result of blood contaminating the CSF sample during collection), 3 TNCC per µL, and 20 mg/dL of CSF micro protein. No signs of inflammation or overt cytologic abnormalities have been observed at any of the recorded time points.

The first CSF analysis from dog 2, at age 2.5 months, showed 1 RBC per per µL of CSF, a TNCC of 3 per µL of CSF, and a CSF mirco protein count of 16 mg/dL CSF. The second CSF analysis, at age 4 months, showed 5 RBC per per µL of CSF, a TNCC of 2 per µL of CSF, and a CSF mirco protein count of 13 mg/dL CSF. The third CSF analysis at 5 months showed 0 RBC per per µL of CSF, a TNCC of 6 per µL of CSF, and a CSF mirco protein count of 16 mg/dL CSF. Normal to mild mononuclear pleocytosis was observed at the 5-month time point.
For both dogs currently enrolled in the study, neurological and physical examinations were conducted weekly, beginning at approximately 12 weeks of age. From 12 weeks of age through approximately 25 weeks, dog 1 was assessed as “normal” per the neurologic exam: mentation was alert, no involuntary movement was observed, gait was normal, postural reactions were intact, cranial nerves were normal, reflexes were intact, and no spinal hyperesthesia was observed. Beginning at approximately 26 weeks of age, the dog developed very mild to mild paraparesis in its gait, while the remainder of the exam was unremarkable. Beginning at approximately 29 weeks of age, the dog began to develop proprioceptive ataxia along with the preexisting mild paraparesis in its gait. At 33 weeks, the dog was assessed as neurologically worsened, with paraparesis and pelvic limb proprioceptive ataxia and thoracic limb cerebellar ataxia, along with decreased tracking in both eyes.

Dog 2 displayed proprioceptive ataxia with mild resistance to neck flexion beginning at approximately 18 weeks. No further neurological symptoms were observed through time of writing (04.03.16).

4.3.2. MRI

At approximately 3 months of age, dog 1 had a calculated ventricle volume of 515.4 mm$^3$, which increased to 909.1 mm$^3$ by approximately 5 months of age and to 1369.4 mm$^3$ by approximately 7 months. Dog 2 had a ventricle volume of 342.6 mm$^3$ at 3 months of age, which increased to 531.2 mm$^3$ by approximately 5 months. In comparison, CNS untreated dogs from the retinal ex...
vivo gene therapy study (Chapter 3, dogs 5 and 6) had ventricle volumes of 702.8 mm$^3$ and 785.1 mm$^3$ at approximately 3 months of age, which later increased to 6391.4 mm$^3$ and 3985.5 mm$^3$ by 9 months.

4.3.3. T-Maze

Dogs 1 and 2 began running the T-maze at 4 months of age, with the initial testing period running from 12.31.15 to 01.12.16 (dog 1) and 02.02.16 to 02.18.16 (dog 2). During this period, dog 1 registered an average 9.33 incorrect choices before first correct choice (IBFC) and 1.33 average incorrect choices after the first correct choice (IAFC) for a total of 10.67 while dog 2 averaged 3.00 IBFC and 0.67 IAFC for a total of 3.67. At 5 months of age, during the testing period running from 01.25.16 to 02.04.16 (dog 1) and 03.02.16 to 3.11.16 (dog 2), dog 1 had an average of 2.00 IBFC and 2.00 IAFC, for a total of 4.00 while dog 2 had an average of 3.00 IBFC, 1.00 IAFC, and a total of 4.00. At 6 months of age, during the testing period from 02.22.16 to 03.05.16 (dog 1), dog 1 had an average of 3.67 IBFC, 4.33 IAFC, and 8.00 total (Figure 4-1).
Figure 4-1. T-maze performance of dogs 1 and 2 as of this writing. The data at 4 and 5 months represent the average of the 2 dogs whereas the data at 6 months is from dog 1 alone since dog 2 has not yet completed this time point. The improvement between 4 and 5 months of age is similar to that seen in normal dogs, whereas the decrease in performance between 5 and 6 months of age is similar to that seen in untreated affected dogs (Katz et al., 2014).

4.4. Discussion

In this study we are attempting to adapt the ex vivo gene therapy approach (outlined in Chapter 4), developed for the sustained delivery of the TPP1 pro-enzyme to the retina for the treatment of CLN2-related retina degeneration, to the central nervous system (CNS) similarly as a means of providing the TPP1 enzyme to the CNS long-term in the TPP1<sup>−/−</sup> long-haired miniature Dachshund model. At the time of writing, these canine studies are still
in their infancy, but initial results suggest that the intracerebroventricular and intracisternal implantation of transduced MSCs should be safe as a long-term CNS delivery method for TPP1 in the treatment of CLN2-related neuronal degeneration. Too little data is available as of yet to determine whether the ex vivo gene therapy approach is more effective in ameliorating the CLN2-related neuronal degeneration versus already established approaches, namely enzyme replacement therapy and conventional gene therapy. However, the lack of any evidence of the adverse effects of this treatment is so far promising.

Neurologic signs have so far been promising in the two current dogs and at no point do they appear to have developed inflammation within the CNS as a result of the intracerebroventricular or intracisternal injection. In a previous study conducted by the lab, a high percentage of dogs receiving AAV2-TPP1 gene therapy injections within the brain and targeted at addressing CLN2-related neuronal degeneration developed severe inflammation and meningitis as a result of the treatment. Elevated cell and micro protein counts were observed in CSF samples taken from these dogs. In contrast, the current ex vivo gene therapy dogs have CSF cell and micro protein counts well within normal ranges and have displayed no signs of either inflammation or meningitis. While still early in these dogs’ treatment course, these results suggest that at least with regards to safety, the ex vivo gene therapy approach is more viable than conventional directed gene therapy. Dog 1 developed very mild paraparesis at 25 weeks of age that progressively worsened to include paraparesis and pelvic limb proprioceptive ataxia and thoracic limb cerebellar ataxia by 33 weeks, while dog 2 developed
proprioceptive ataxia at 18 weeks of age, but otherwise has remained neurologically normal. Again, while still very early, these neurologic results are at least initially quite promising.

Similarly to the current absence of neurologic signs in the treated dog, initial MRI and T-maze results are also very promising. Untreated dogs (dogs enrolled in the retinal studies in Chapter 4) developed severe progressive ventricular enlargement: at 3 months of age, both reference dogs had ventricle volumes of over 700 mm$^3$, while at the same age the CNS treated dogs had volumes of only 515.4 mm$^3$ and 342.6 mm$^3$. By 9 months, the untreated dogs had ventricle volumes of approximately 4000-6000 mm$^3$. While the CNS treated dogs are only 7 and 6 months old at the time of writing, and their most recent MRIs were at 7 and 5 months of age, they had only progressed to a volume of 1369.4 and 531.2 mm$^3$, respectively. As mentioned previously, it is still too early to draw any meaningful conclusions and make any predictions with regards to the dogs’ future results, but the initial data do suggest that the CNS treatment is at least delaying ventricle enlargement in both dogs relative to untreated dogs.

The dogs also showed improvement when subjected to the T-maze over subsequent months, with an initial averaged improvement similar to that seen in normal dogs. The decrease in performance by dog 1 at 6 months of age is consistent with affected untreated dogs. Two and three time points per dog are insufficient to mark a trend, but the improvement month over month observed is initially suggestive that the treatment is having a beneficial effect on the dogs’
cognitive faculties, though the treatment dose may need to be increased in magnitude.

The initial neurologic, MRI, and T-maze results from these first two dogs suggest that the AAV2-TPP1 transduced MSCs may able to continuously supply the TPP1 pro-enzyme to the CSF and circulate it within the central nervous system environment. Based on the results of the, so far, two treated dogs versus data from untreated controls, it appears that the cells may be sufficient enough in number so as to supply a base level of TPP1 to the cells within the CNS such that it is able to exert a neuroprotective effect. As of this writing, no deleterious side effects were observed as a result of the treatment in the CNS/CSF. As a result of the apparent initial success of the *ex vivo* gene therapy approach in these first two dogs in ameliorating the CLN2-related neuronal degeneration, additional dogs will be enrolled in the study to better investigate the safety and efficacy of this approach within the CNS environment.
CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

We have developed an *ex vivo* gene therapy approach for the sustained delivery of tripeptidyl peptidase-1 (TPP1) to the retina of CLN2 affected dogs to delay disease-related progressive retinal degeneration and blindness. Studies utilizing this approach to treat CLN2-realted central nervous system (CNS) degeneration are currently ongoing.

Using this approach, we have documented clear therapy-dependent attenuation of CLN2-related retinal degeneration in affected dogs. Dogs that received the *ex vivo* gene therapy treatment displayed marked preservation of their b-wave ERG responses relative to their control eyes, which is indicative of preservation of the cells within the inner retina, though there did not appear to be as high a degree of preservation in the a-wave responses, which gauge the health of the photoreceptors within the outer retina. However, treated eyes displayed b:a-wave ratios comparable to normal, unaffected dogs, suggesting that the inner retina is functioning normally in proportion to the stimulus it is receiving from the photoreceptors. Furthermore, the multifocal retinopathy observed in the treated eyes was much less severe than in the contralateral control eyes, either with a delayed presentation and lower grade by disease end-stage or by a failure to develop retinopathy at all.

Variable numbers of cells were injected during the course of these studies (“dosages”) with degree of preservation appearing to be dose-dependent, though the data suggest that there is an upper threshold above which there does not
appear to be an appreciable difference in preservation. ERG responses initially appeared to simply display a presence/absence response to the treatment: dogs that received a lower dose (approximately 2.5 million cells) displayed similar high intensity cone and rod response preservation to dogs that received higher doses (9 to 12.5 million cells) through 9 months of age. By 10 to 11 months of age (disease end-stage), the high dose dogs were still displaying higher responses in their treated eyes versus control while the lower dose dogs showed similar degeneration in both eyes. Dogs that received a higher dose showed similar ERG responses in their treated eyes regardless of number of cells injected beyond approximately 9 million. It may be possible that additional cells beyond a 12.5 million cell dose may be able to extend the observed preservation and may be able to reduce the significance of degeneration month to month.

Similarly, high dose dogs displayed reduced or delayed canine multifocal retinopathy, with less severe presentation versus the low dose dogs. However, there was variability in the degree of preservation among the high dose dogs, with those dogs receiving the highest cell dosage not necessarily being the dogs that did not develop the retinopathy at all. Across all dogs for both the ERG and retinopathy data, the dog that displayed the greatest degree of retinal preservation received a dose of 10 million cells, which was not the highest dose injected. As with any other treatment, it does appear that some variability on a patient-by-patient basis exists, even in a model with an identical causative mutation underlying the disease.
A combined treatment regimen is currently in progress, utilizing the \textit{ex vivo} gene therapy approach to treat both the retina, via intravitreal injection, and the CNS, via intraventricular and intracisternal injection. Previous work conducted by the lab illustrated that TPP1 enzyme replacement therapy directed towards the cerebrospinal fluid is effective in delaying CNS degeneration and prolonging life. These current studies are attempting to replicate these results using the \textit{ex vivo} gene therapy approach instead, as it ideally would require a single high cell dose (~160-200 million cells currently utilized in these studies) versus the biweekly infusions utilized in the enzyme replacement approach. If successful, preservation of the CNS would allow for the determination of the extent to which a single intravitreal injection may preserve retinal function, as well as allow repeated treatment of the retina beyond ages currently possible due to disease end-stage.

We have been issued a provisional patent for our \textit{ex-vivo} gene therapy approach for sustained delivery of therapeutics to the retina and CNS. This technology has tentatively been designated CellGenRx for which we are seeking trademark protection. A manuscript describing our finding with the eye treatment is currently being prepared for submission to Science Translational Medicine. We hope to license the CellGenRx technology to a company that will take this to clinical trials for CLN2 disease.
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