Maze testing of sheep for early detection of Batten disease

A thesis submitted in fulfilment of the

requirements for the Degree of Master of Science

at

Lincoln University

by

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2020

Abstract of a thesis in fulfilment of the requirements for the degree of MSc.

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Batten disease is a group fatal inherited neurodegenerative diseases typically manifesting in humans in childhood. The diseases are genetically heterogeneous and characterised by cognitive loss, psychomotor deterioration, retinal degeneration, brain atrophy, seizures and premature death. Treatments for Batten disease are at an early stage, but this study is part of the development of gene therapy for the disease caused by mutations in CLN5 and CLN6.

Many of the forms of Batten disease that occur in humans have also occurred spontaneously in large animals, including ovine species. Two naturally occurring sheep models of NCL are maintained at Lincoln University, a CLN6 form in South Hampshire sheep and a CLN5 form in Borderdale sheep. Affected sheep are normal at birth, but develop clinical symptoms at around 10-14 months of age. These include progressive loss of sight, and psychomotor decline, as a result of severe cortical loss and loss of retinal photoreceptors. Premature death generally occurs at around 2 years of age.

Cognitive decline is a common symptom of human Batten disease and also occurs in the CLN5 and CLN6 ovine forms of the disease. The purpose of this study was to investigate if a closed-field maze test could be a useful method for determining loss of cognition in sheep with Batten disease. It was hypothesised that cognitive decline may affect an affected sheep's ability to transit a mazes, hence offering a potential method for early diagnosis of the disease. This would help speed up the optimisation of therapeutic interventions, by providing an early measure of their efficacy.

The study consisted of three experiments. The first experiment involved the development of a maze that was navigable by the sheep, and complex enough to discern between normal and affected animals. Time to complete, and path length, determined by high precision GPS, were both used as measures of the sheep's ability. The second experiment used the final iteration of the maze

developed in experiment one, to determine when differences could be seen between normal and affected animals, and measure the effectiveness of the gene therapy treatment. The final experiment used a more cognitively complex maze that used visual cues to indicate the correct path through the maze.

This study established that sheep were able to navigate a complex maze. The performance of the normal cohort of both breeds was the same. All animals were slowest and took their longest paths when first exposed to the maze, but were faster and took shorter paths in subsequent transits. The untreated affected cohorts of both genotypes were generally slower and took a longer path length through the maze than their unaffected counterparts. The loss of ability of individual affected (both untreated and treated) animals to negotiate the maze correlated well with other measures of disease progression. Due to the very variable performance of affected and treated animals, the experiment did not achieve the objective of being able to discern the onset of Batten disease at an earlier stage than other measures currently in use.

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1 Introduction

Batten disease is a group of diseases referred to as neuronal ceroid lipofuscinoses (NCLs). This is a group of fatal inherited neurodegenerative diseases typically manifesting in humans in childhood. The NCLs are classified as rare diseases, and thought to have an incidence of about 1:12,500 births (Rider & Rider, 1988). The diseases are genetically heterogeneous, with defects in any one of 13 genes, designated *CLN1-8* and *10-14* that cause NCL. As a group, they are characterised by cognitive loss, psychomotor deterioration, retinal degeneration, brain atrophy, seizures and premature death (Mole, Williams, & Goebel, 2005). At a cellular level the diseases are characterised by a widespread accumulation of protein, either subunit c of mitochondrial ATP synthase or saponins A and D, in lysosome derived storage bodies (Palmer, 2015). Treatments for Batten disease are at an early stage, but this study is part of the development of gene therapy for the disease caused by mutations in CLN5 and CLN6.

Many of the forms of Batten disease that occur in humans have also occurred spontaneously in large animals, including ovine, bovine, caprine, canine, equine, feline and porcine species (Bond, Holthaus, Tammen, Tear, & Russell, 2013). Affected sheep make useful models, as they demonstrate many of the clinical and pathological symptoms found in the human diseases. Their gyrencephalic brain is similar in organisation to the human brain, and is of a convenient size for potential therapies. Sheep are amenable to handling and can be kept at a low cost. Two naturally occurring sheep models of NCL are maintained at Lincoln University : a CLN6 form in South Hampshire sheep and a CLN5 form in Borderdale sheep (Jolly, Arthur, Kay, & Palmer, 2002; Jolly RD, 1975). Affected sheep are normal at birth, but develop clinical symptoms at around 10-14 months of age. These include progressive loss of sight, and psychomotor decline, as a result of severe cortical loss and loss of retinal photoreceptors. Premature death generally occurs at around 2 years of age.

All different forms of Batten disease stem from the loss of gene function, therefore gene therapy would appear to be a promising form of treatment of the disease. In this study self-complementary adeno-associated viral vectors (scAAV) were used to deliver a normal functional copy of the CLN5 and CLN6 Batten disease genes. Treatment efficacy can be judged by longitudinal neuroimaging to measure brain atrophy, and clinical scoring using Batten disease rating scales to measure cognitive, vision and motor decline. Human assessment methods have been adapted for use in ovine forms of the disease (Mitchell et al., 2018; K. N. Russell et al., 2018).

The current studies in the ovine forms of CLN5 and CLN6 Batten disease are using longitudinal computed tomography (CT) imaging to measure any decline in intracranial volumes as an indicator of brain atrophy, electroretinography (ERG) to measure retinal degeneration, and a range of assessments included in the ovine Batten disease rating scale (oBDRS). The oBDRS consists of physical assessments of visual and auditory function, posture, movement, body tremors and body condition scoring, as well as behavioural assessments of mentation, aggression and capability/independence (Mitchell et al., 2018).

Cognitive decline is a common symptom of human Batten disease (Anderson, Goebel, & Simonati, 2013) and also occurs in the CLN5 and CLN6 ovine forms of the disease (Mitchell 2018, 2019). The purpose of this study was to investigate if a closed-field maze test could be a useful method for determining loss of cognition in sheep with Batten disease. It was hypothesised that cognitive decline may affect an affected sheep's ability to transit a mazes, hence offering a potential method for earlier diagnosis of the disease than is possible with current methods. This would help speed up the optimisation of therapeutic interventions, by providing an early measure of their efficacy. To this end untreated Batten diseased sheep, sheep with Batten disease undergoing gene therapy treatments, and healthy normal sheep were tested in a variety of mazes. Some of the animals used were involved in other studies, hence underwent concurrent

computed tomography scanning and electroretinography to monitor brain atrophy and retinal function respectively, and a range of other non-invasive tests to assess their disease progression.

1.1.1 History of the field maze and the two choice maze

The use of a closed field maze to test the 'mental' ability of rats was first published in 1946 (Hebb DO, 1946). This field maze consisted of a square arena with an entrance in one corner and food reward in the opposite corner. The path between the corners was obstructed by panels to create a maze, and the animal was scored on the number of times it entered error zones. Twelve different formats of the maze were used to test each animal. This maze was modified (Figure 1-1) (Rabinovitch, MS., 1951) and divided into 36 squares and this formed the grid pattern for the placement of the barriers. A set of 6 relatively simple training problems was developed, and a further set of 12 more complex test mazes. The Hebb-Williams maze is still being used today and has been used to test rats, cats, rabbits, ferrets, possum, mice, voles, sheep, rams, lambs, goldfish, leghorn chicks, bears, raccoons, baby monkeys, cows and even humans (Shore, Stanford, MacInnes, Brown, & Klein, 2001). This form of maze is primarily designed to measure spatial memory and learning.

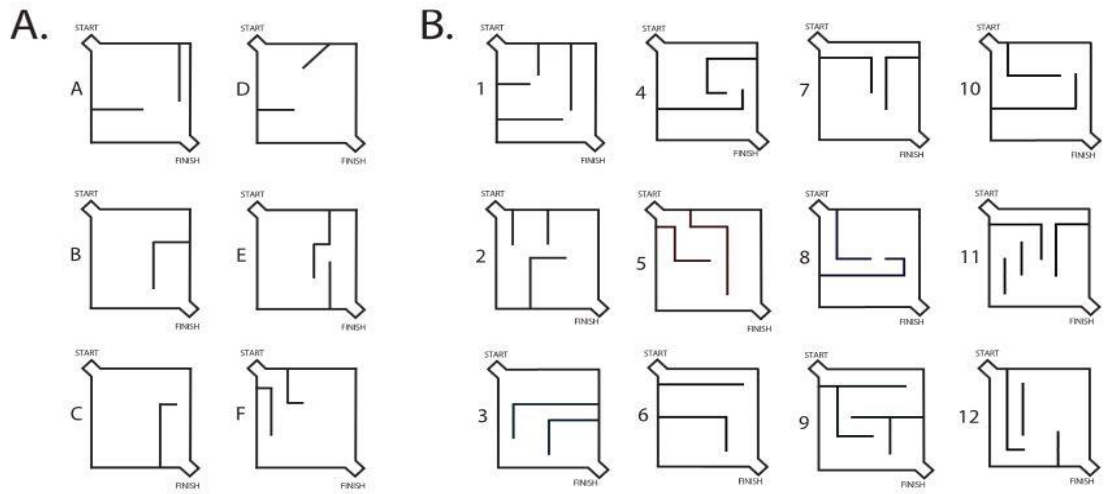

Figure 1-1. The six acquisition mazes (A-F), and 12 testing mazes in the Hebb-Williams maze as modified by Rabinovitch (1951).

The first records of the Hebbs–Williams maze in use with large animals were with sheep (Kilgour, R.; Bruere, 1970) and dairy cows (Kilgour, 1981). The majority of work with such a maze has been in rats, and scaled up versions have been used to test sheep (Lee, Colegate, & Fisher, 2006), cattle (Hirata, Tomita, & Yamada, 2016; Kilgour, 1981), pigs (Jansen, Bolhuis, Schouten, Spruijt, & Wiegant, 2009) and dogs (Elliot & Scott, 1965). The experimental use of mazes has revealed that sheep and other large herbivores have excellent spatial cognition and memory. It is thought that, as animals that would naturally graze over extensive areas, this ability has allowed them to return to particular food sources repeatedly over time (Dumont & Petit, 1998; Edwards, Newman, Parsons, & Krebs, 1997). The ability to traverse a maze has been studied with sheep subjected to negative stimuli such as images of dogs or white noise (Doyle, Freire, Cowling, Knott, & Lee, 2014). As testing of large animals in mazes has increased, the recognition of their capabilities has become apparent, their usefulness as models of many diseases has also been realised.

The two choice maze was also investigated in this study. In this maze, the animal has a choice between two paths; the correct path leading to a food reward, or passage through the maze. This form of maze is generally accepted to have originated in the USA in the 1890s, for use with rats. Rodents, particularly rats, are still the main subject for this type of maze, but it is now more commonly being used in large animal studies. Examples include studying learning, cognition, drug effects, and mental states. These mazes are generally in the form of a T shape (Camm, Gibbs, Cock, Rees, & Harding, 2000; Johnson, Stanton, Goodlett, & Cudd, 2012; Taylor, Brown, Price, & Hinch, 2010) or a Y (Ferreira, Keller, Saint-Dizier, Perrin, & Lévy, 2004; Hernandez et al., 2009; Hunter et al., 2015; Kendrick et al., 1995; Peirce, Leigh, Dacosta, & Kendrick, 2001) , giving a two choice option. This format is useful to test the ability of an animal to learn to associate symbols, colours or pictures with food rewards or passage through the maze. The ability of sheep to learn these associations, and then learn reversal of the association has been tested thoroughly (Hunter et al., 2015; Johnson et al., 2012; McBride, Perentos, & Morton, 2016; Morton & Avanzo, 2011;

Taylor et al., 2010). This ability has been tested when animals that have been exposed to intrauterine undernutrition (Camm et al., 2000; Erhard, Boissy, Rae, & Rhind, 2004; Hernandez et al., 2009), and used to study foraging strategies (Hosoi, Swift, Rittenhouse, & Richards, 1995).

Our understanding of the cognitive capacities of sheep has increased enormously in the last few years. The increased use of large animal models in medical research, and an increased awareness of animal welfare has caused an increase in studies into cognition and learning abilities. It is becoming increasingly obvious that the public perception that domesticated ungulates are "stupid" is misplaced. Through the use of mazes we now know that they are capable of recognition of colours, symbols and faces, both of their own species and humans. Once learned we know that the animals can reverse their learning, or shift their learning from one dimension to another i.e. learning a coloured bucket, then shifting to the same coloured cone. We also know that once learned, sheep can retain this knowledge for long periods of time, up to at least 2 years in the case of recognition of human faces (Keith, Ana, Andrea, Michael, & Jon, 2001). Cognitive tests for use on sheep have become more and more sophisticated as the ability of the sheep is better understood. One group of researchers (McBride et al., 2016) have developed a semi-automated mobile system that has LCD screens to display symbols, and an automatic food reward dispenser that dispenses food when the animal approaches the correct screen. This system has then been used to trial stop signals (Knolle, McBride, Stewart, Goncalves, & Morton, 2017) and stop signal reaction time. The work demonstrated that sheep are able to negotiate a two choice maze, a complex cognitive task, and are able to stop the response 91% of the time. The drawback to this level of complexity is the time taken to conduct the trials. The study of the stop signal task (Knolle et al., 2017) required about 300 runs/animal of training, and 450 runs for the experiment. These were animals that had also historically been used in other cognition trials by the same group (McBride et al., 2016; Morton & Avanzo, 2011), so were somewhat conditioned to this form of experiment before they started.

Despite this knowledge of the cognitive capabilities of animals, studies that use these testing systems in an applied way are limited. As previously described, mazes have been used in a few studies on sheep on the effects of prenatal undernutrition, and also the effect of stressors such as dogs or white noise. There do not appear to be any studies using maze ability to measure disease progression in sheep. A T-maze has been used to study cognitive decline in a dog model of CLN2 Batten disease (Sanders et al., 2011), and was found a significant difference between normal and affected animals at an earlier stage than any other clinical indicators the researchers were using.

In the current study we started with a simple field maze, in a rectangular format, with a series of gates to be negotiated for the animal to get to conspecifics housed at the far end. The maze was developed during the course of the study to increase its complexity whilst retaining its practicality.

2 Development of a closed-field maze

2.1 Materials and methods

2.1.1 Animals and experimental design

This maze study was conducted on 22 animals in accordance with the NZ Animal Welfare Act (1999) and approved by the Lincoln University Animal Ethics Committee (LUAEC #614). All trial sheep were first tested at 8.5 months of age (range min 7.6, and max 8.9) in four different cohorts

The normal control cohort consisted of three South Hampshire CLN6$^{+/-}$ and three Borderdale CLN5$^{+/-}$ sheep. The affected cohort consisted of three South Hampshire CLN6$^{-/-}$ and three Borderdale CLN5$^{-/-}$ sheep. The first treated cohort consisted of six South Hampshire CLN6$^{-/-}$ sheep who received intracerebroventricular delivery of either scAAV9.CLN5 or scAAV9.CLN6 at 3 months of age. The second treated cohort consisted of four Borderdale CLN5$^{-/-}$ sheep who received intracerebroventricular delivery of scAAV9.CLN5 at 7 months of age. All 22 animals were part of other projects within the Batten disease research project and hence received CT scans and electroretinography every 2-3 months throughout their lifetime (Mitchell et al., 2018; K. N. Russell et al., 2018). Animals were grazed on grass/white clover pasture with water available *ad libitum*.

Sheep #	Genotype	Treatment	Other *in vivo* assessments
1001	$CLN6^{+/-}$	None, normal control	CT, ERG (Russell 2017, Russell et al., 2018)
1004	$CLN6^{+/-}$		
1005	$CLN6^{+/-}$		
1100	$CLN5^{+/-}$		
1105	$CLN5^{+/-}$		
1106	$CLN5^{+/-}$		
1008	$CLN6^{-/-}$	None, affected control	CT, ERG (Russell 2017, Russell et al., 2018)
1014	$CLN6^{-/-}$		
1109	$CLN5^{-/-}$		
1110	$CLN5^{-/-}$		
1122	$CLN5^{-/-}$		
1033	$CLN6^{-/-}$	scAAV9.CLN5@ 3 months	CT, ERG
1040	$CLN6^{-/-}$		
1038	$CLN6^{-/-}$	scAAV9.CLN6@ 3 months	
1042	$CLN6^{-/-}$		
1045	$CLN6^{-/-}$		
1164	$CLN5^{-/-}$	scAAV9.CLN5 @ 7 months	CT, ERG (Mitchell et al., 2018)
1165	$CLN5^{-/-}$		
1170	$CLN5^{-/-}$		
1172	$CLN5^{-/-}$		

Table 2-1. List of animals used in Experiment 1.

2.1.2 Maze

A simple closed field maze was utilised at approximately monthly intervals to assess the visual and cognitive faculties of normal (heterozygous), affected, and affected sheep of both genotypes that had received gene therapies. It was constructed in a 5.8m wide lane between two 5 wire fences. The ends of the maze, and all internal dividers were tubular metal gates with wire mesh infills which allowed uninterrupted sight for each individual test subject through the maze to conspecifics housed in an end pen (Figure 2-1). When an animal was released from the holding pen, it was subject to two drivers that made it traverse the maze, the desire to get away from the operator, and the desire to be reunited with its conspecifics. Each animal was tested in a sequence of either 4 or 5 consecutive runs.

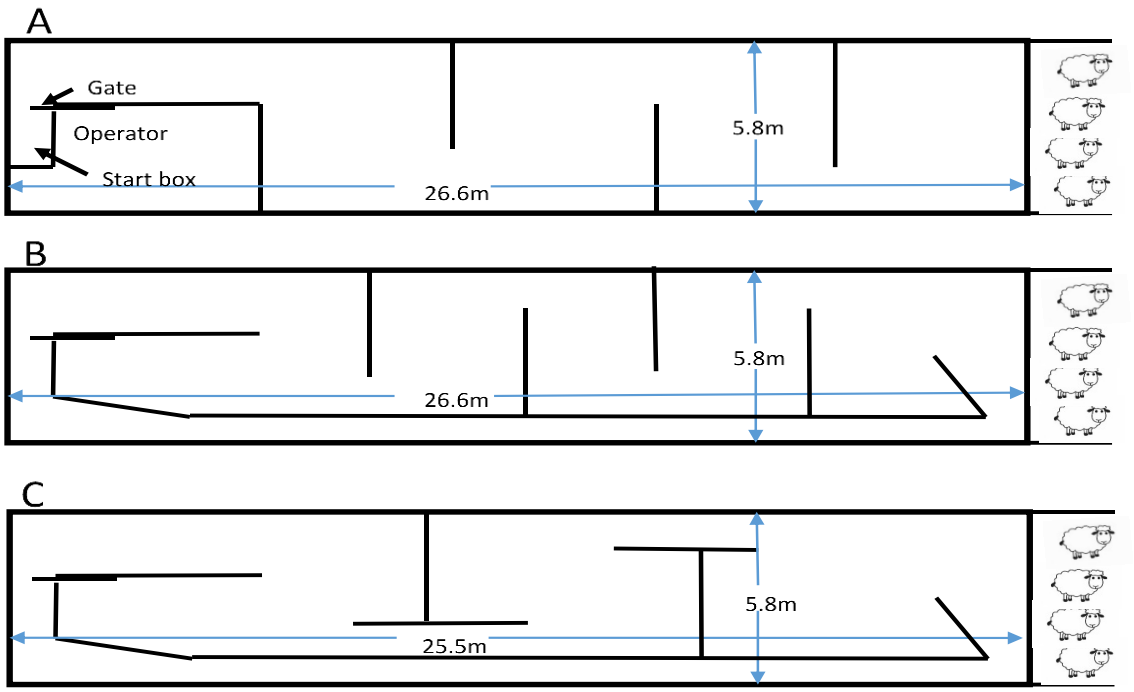

Figure 2-1. Layout of first three mazes.

The configuration of the maze was adapted over the course of the study. The initial maze consisted of a series of three gates over a distance of 26.6 metres (Figure 2-1 A), and the animals were first tested at a mean age of 8.5 months. The final maze alteration was made when the animals were tested at 16 months of age (Figure 2-2). An extra gate to negotiate was included, with an extra error space, and the maze was shortened by 5 metres to bring the conspecifics closer, presenting a stronger draw for the test animal. This also brought the conspecifics out of the shade of a wooded shelter belt, and hence they were more visible to the test animal. The position of the operator did not change during any of these maze iterations, and the ground was sprayed with glyphosate when required to keep it free of plant material which could be a distraction for the sheep.

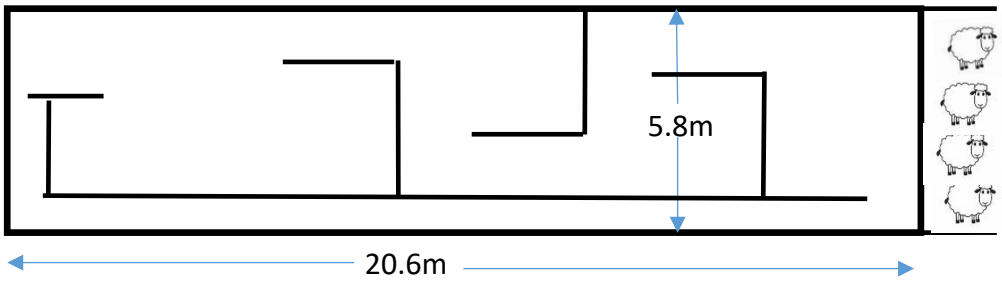

Figure 2-2. Final maze layout for Experiment 1.

2.1.3 GPS tracking

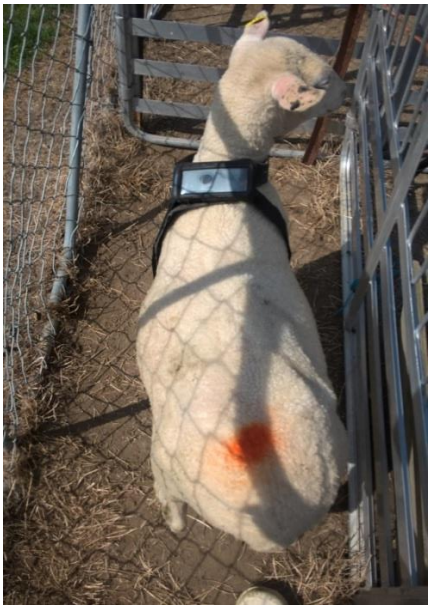

Figure 2-3. Animal with GPS tracker unit attached.

Animals wore a simple Velcro (50mm wide) home–made harness during testing, consisting of a neck and chest strap (*Figure 2-3*). A GPS tracker (Trimble R1, Trimble Inc, Sunnyvale, CA.) was attached to this, set to record at a frequency of 1Hz. It was operated via a Bluetooth connection to a laptop located beside the operator at the start of the maze. Data was processed by Trimble Terrasync Software (v5.90), and then sent to AllTerra NZ (Addington, Christchurch, NZ), for centimetre level post-processing. Each test subject was held in the start pen whilst the GPS software was initiated before the start of each maze run, and then for a further 15 seconds to allow the starting point to be identified in the GPS data (the first recording where either the Easting or Northing position moved by more than 20cm).

To back up the GPS data the time taken from exiting the start pen, to passing the final gate, was also recorded manually with a stopwatch by the operator. The cut off point for the time was 20m through the maze in the first 3 maze iterations, when it was 25m long. The final maze was only 20m long and the cut-off point was 15m, or when the animal passed the last gate. Animals were allowed 120 seconds to complete the maze, otherwise they were judged to have failed. When that happened they were pushed through by the operator, and given a recorded time of 120 secs. Each maze test session initially comprised of 4 runs, but from 9.4 months of age (min 8.5, max 9.8) all animals were tested over 5 runs. Average times for the 4-5 runs were reported, with animals failing all 4-5 runs given an average time of 120 secs. All animals were tested in blocks at approximately monthly intervals.

2.1.4 Gene therapy

The gene therapy treatment involved intracerebroventricular delivery of a gene therapy vector, self-complementary adeno-associated virus serotype 9 (scAAV9) expressing either the ovine CLN5 or CLN6 genes. In brief, under stereotaxic guidance, a total volume of 400 µl of vector was injected into each cerebral lateral ventricle (Mitchell et al., 2018). The first treated cohort consisted of four Borderdale CLN5$^{-/-}$ sheep who received 4.0 x 10^{12} vg (viral genomes) of scAAV9.CLN5 at 7 months of age. Six South Hampshire CLN6$^{-/-}$ sheep in the second cohort received either 4.6 x 10^{12} vg of scAAV9.CLN5 (n=3) or 3.5 x 10^{12} vg of scAAV9.CLN6 (n=3) at 3 months of age.

2.1.5 Electroretinography (ERG)

All animals involved in this trial also received two monthly electroretinography as part of a separate trial to test retinal function (K. Russell, 2017). This was conducted using an Eickemeyer Veterinary ERG system (Eickemeyer -Medizintechnik für Tierärzte KG, Tuttlingen, Germany) and measured the action potential generated by the retina in response to a flash of light.

Measurements were conducted under conditions of ambient light, in the dark (dark adapted), and after being in the dark for five minutes (5 mins dark adaption). The amplitude data for the 5 mins dark adapted a and b waves were then compared to the maze traverse times for these animals.

2.1.6 Intracranial volume (ICV)

Intracranial volume has been proven as an accurate surrogate for brain volume in ovine NCL (K. N. Russell et al., 2018). Intracranial volume data were collected for all animals in the present study as part of other concurrent trials (Mitchell et al., 2018; K. N. Russell et al., 2018). Volume measurements were inferred from three-dimensional reconstructions of the cranium from longitudinal computed tomography (CT) scans by established methods (K. Russell, 2017).

2.1.7 Statistical techniques

Means and corresponding SEM was calculated for each group at each time point. Student's t-tests were performed to test each group against the normal controls at each time point. Differences were regarded as significant where $P<0.05$. Where individual treated and untreated affected animals were compared to the control group, they were considered to be significantly different from controls when they were more than 1.96 standard deviations away from normal control mean values.

2.2 Results

2.2.1 Heterozygous normal control animals

Mean traverse times for heterozygous normal control sheep of each breed were plotted (Figure 2-4). Times were not significantly different ($P>0.05$) for the unaffected $CLN5^{+/-}$ and $CLN6^{+/-}$ control groups at any time over the whole experiment, but there was a trend for the Borderdale $CLN5^{+/-}$ to be slower than the South Hampshire $CLN6^{+/-}$. More variation was observed in the traverse

times of the CLN5$^{+/-}$ cohort, who would often stop and stand still towards the end of maze. In comparison the CLN6$^{+/-}$ sheep exhibited a stronger desire to get away from the operator, and associate with conspecifics.

Over the course of the experiment the traverse times for the CLN6$^{+/-}$ cohort gradually slowed (Figure 2-4), and were significantly ($P<0.05$) slower from 14 months onwards than they were at the start of the experiment. The more variable CLN5$^{+/-}$ cohort showed no significant change in traverse times over the course of the experiment.

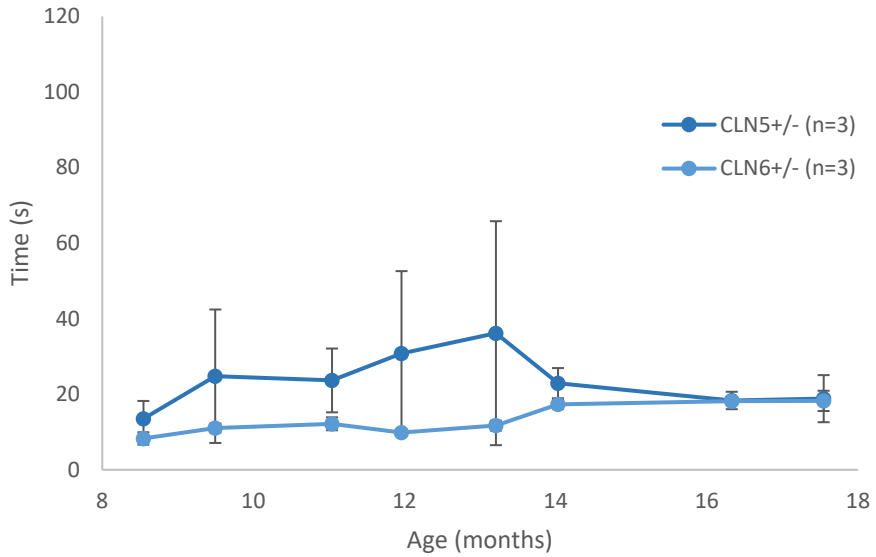

Figure 2-4. Mean (+/- sem) maze traverse times for normal CLN5$^{+/-}$ and CLN6$^{+/-}$ cohorts.

Whilst the path through the maze taken by individual animals within the two control cohorts did not vary significantly ($P>0.05$), their times between 9-13 months of age frequently did. Figure 2-5 shows the spatial paths of a normal control CLN5$^{+/-}$ and CLN6$^{+/-}$ animal at 13 months of age. Although the path is very similar, the average time taken by the CLN5$^{+/-}$ animal was significantly longer ($P<0.05$, mean time of 65s for the CLN5$^{+/-}$ vs 8.9s for the CLN6$^{+/-}$).

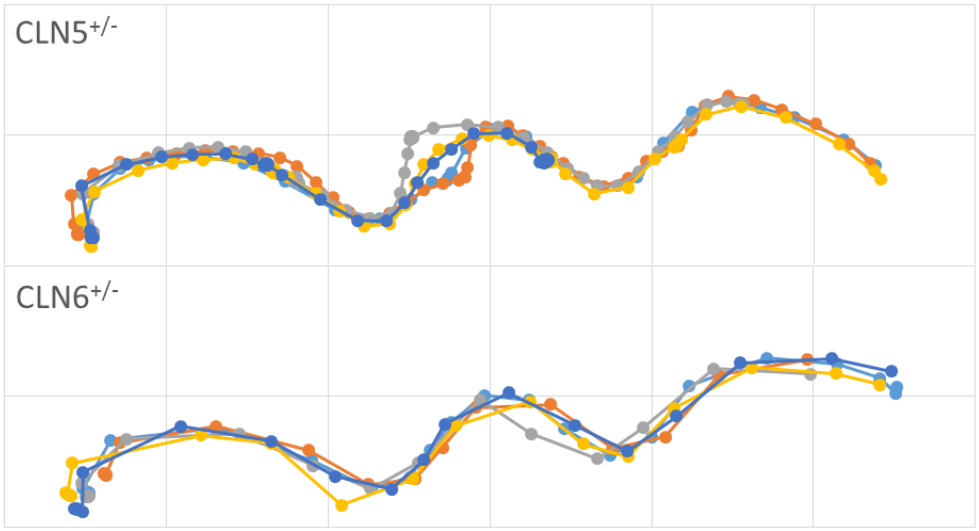

Figure 2-5.Representative spatial plots (collected at 1Hz) of five traverses through the maze by 13 month old normal control CLN5$^{+/-}$ and CLN6$^{+/-}$ animals. The CLN5$^{+/-}$ sheep recorded a mean traverse time of 65 seconds and the CLN6$^{+/-}$ animal a mean time of 8.9 seconds.

2.2.2 Maze traverse times of the normal control and affected cohorts

The mean traverse time for CLN5$^{-/-}$ affected sheep was very similar to the CLN5$^{+/-}$ normal animals, until around 16 months of age (*Figure 2-6*). After that the affected cohort times slowed, although statistical significance was not achieved as comparative data for the normal cohort ended at 17.5 months when these animals returned to the heterozygous breeding flock. All CLN5$^{-/-}$ affected animals failed to negotiate the maze before or at 19.5months.

The pair of affected CLN6$^{-/-}$ sheep were significantly ($P<0.05$) slower than the normal CLN6$^{+/-}$ animals in traversing the maze from 11 months old. One of the CLN6$^{-/-}$ sheep could no longer traverse at 14 months, and the other CLN6$^{-/-}$ sheep failed to complete the maze at 16.3 months (Figure 2-6).

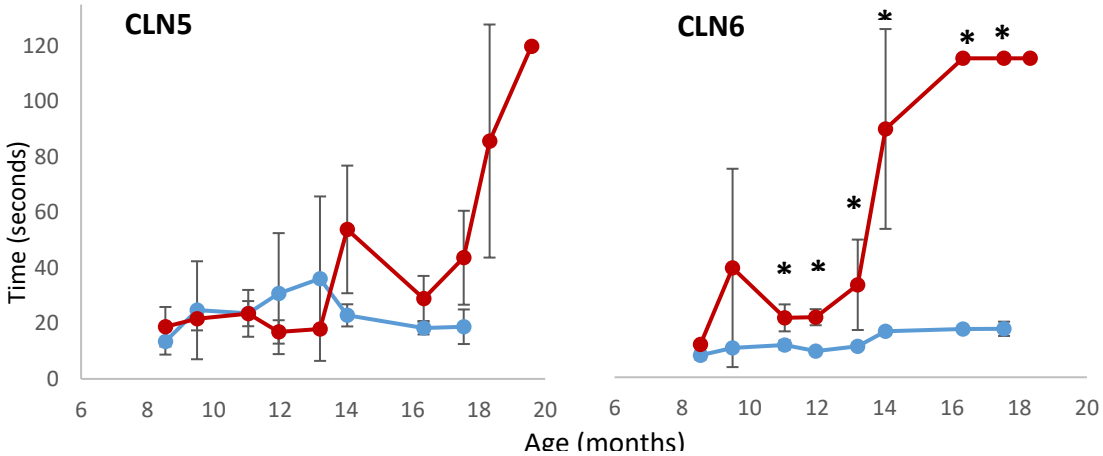

Figure 2-6. Mean (+/- sem) maze traverse times for normal (blue line) and affected (red line) CLN5 and CLN6 cohorts. All cohorts n=3 except CLN6$^{-/-}$ n=2. Asterisk denotes significant difference (P<0.05).

2.2.3 Comparison of maze distance ratios for control and affected cohorts

It was noted that the Borderdale CLN5 sheep had behavioural differences to the South Hampshire CLN6, they appeared more habituated to humans, and had less attraction to their cohort. They would often stop in the middle of the maze, and watch the operator, rather than continue to their conspecifics at the end of the maze. This trait resulted in slow times, despite them having no difficulty in traversing the maze. To remove time spent stationary from the assessment, the GPS track data were used to calculate the distance the animals walked as a proportion of the linear distance through the maze. Figure 2-7 illustrates the GPS tracks over 5 attempts for two animals. The CLN5+/- takes a very direct path through the maze on each attempt, whilst the CLN6-/- animal made several mistakes. Analysis done on a time basis (

Table *2-2*), showed that the CLN6-/- animal had a faster time (mean 50.5s) than the CLN5+/- sheep mean 93.4s) despite the errors made by the former. In comparison, analysis of the distance travelled as a proportion of the linear distance (

Table *2-2*), revealed that the CLN6-/- animal had a higher ratio of distance travelled (1.6 vs 1.38), more accurately reflecting its poorer ability to traverse the maze.

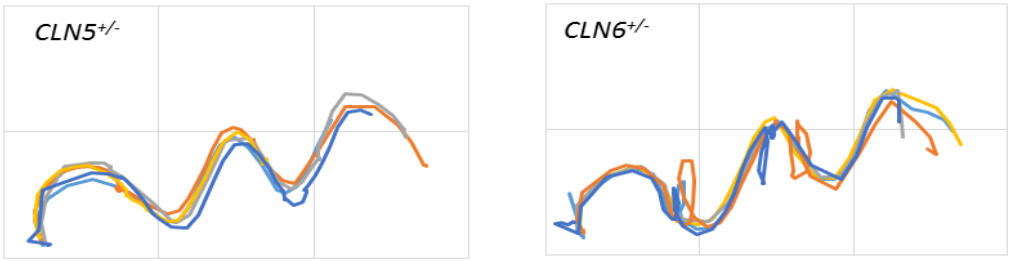

Figure 2-7. Spatial GPS plots (collected at 1Hz) of five traverses through the maze by a single CLN5$^{+/-}$ and CLN6$^{-/-}$ animal.

		$CLN6^{-/-}$	$CLN5^{+/-}$	
Run	*Time (s)*	*Dist (travelled/linear d.)*	*Time (s)*	*Dist (travelled/linear d.)*
1	*34.2*	*1.50*	*54.0*	*1.35*
2	*19.8*	*1.32*	*120.0*	*1.35*
3	*18.8*	*1.30*	*90.8*	*1.38*
4	*120*	*1.95*	*120.0*	*1.35*
5	*59.2*	*1.76*	*82.2*	*1.48*
mean	***50.4***	***1.6***	***93.4***	***1.38***

Table 2-2. Time to traverse and distance travelled as a proportion of linear distance through the maze. Data from five runs from two animals as illustrated in Figure 2-7.

The distance travelled as a proportion of the linear distance was calculated for all four cohorts (*Figure 2-8*) and showed that contrary to the time results (*Figure 2-6*), the variability in the data was greatly reduced. The CLN5$^{-/-}$ sheep went significantly ($p<0.05$) further than their normal CLN5$^{+/-}$ counterparts at the 8.5 and 14 months. The CLN6$^{-/-}$ sheep did not travel significantly ($P>0.05$) further at the 8.5 or 9 month time points, but did when 11 months, and at all remaining ages.

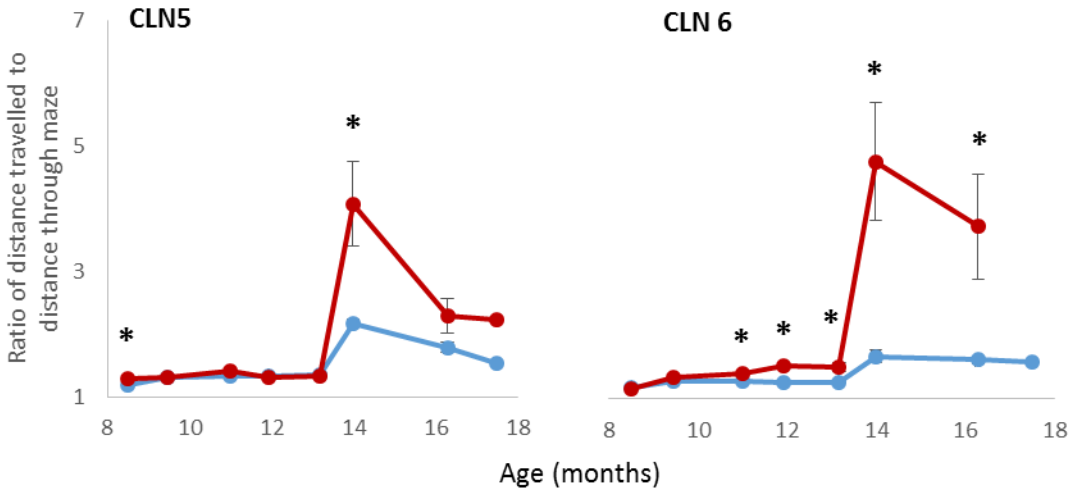

Figure 2-8. Mean (+/- sem) ratio of distance travelled by the animals as a proportion of the linear distance through the maze for normal (blue line) and affected (red line) CLN5 and CLN6 cohorts. All cohort n=3 except CLN6$^{-/-}$ n=2. Asterisk denotes significant difference (P<0.05).

2.2.1 Comparison of maze traverse times and electroretinogram responses for control and affected cohorts

Electroretinography showed that the dark adapted a and b-waves declined steadily for both CLN5$^{-/-}$ and CLN6$^{-/-}$ animals (Figure 2-9) from around 2 months of age. The ability to traverse the maze persisted even with major declines in eyesight, but as the amplitude of the a and b waves approached zero, indicating retinal blindness, then the animals failed to traverse the maze.

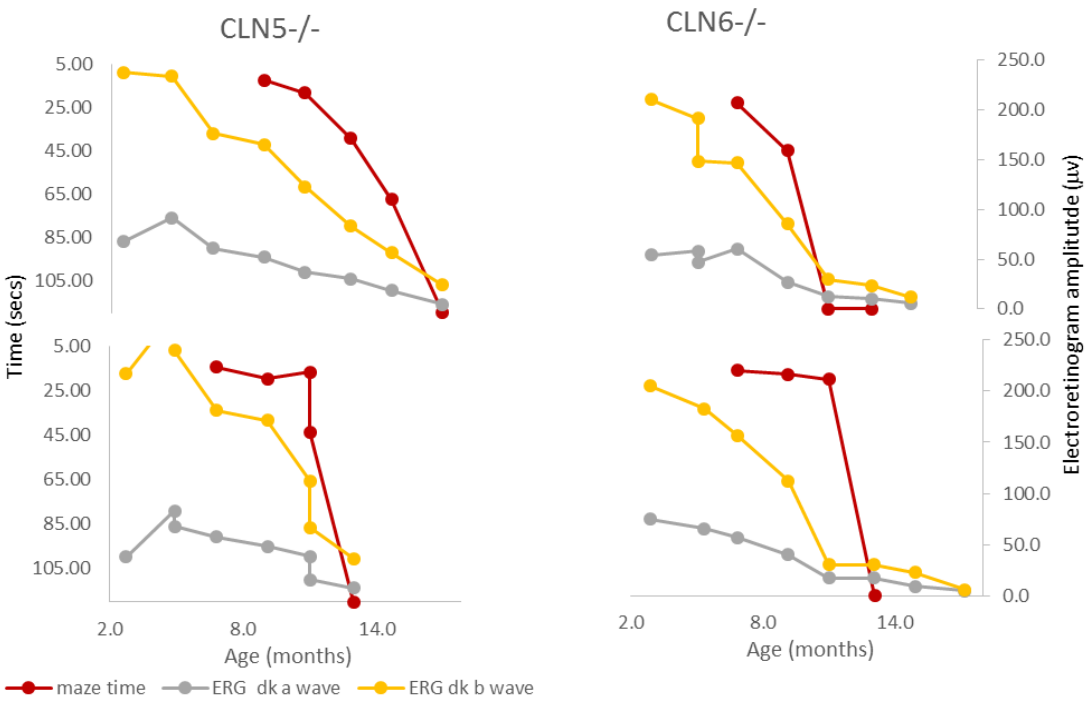

Figure 2-9. Comparison of maze traverse times (red line, primary y-axis), dark adapted a-wave electroretinogram (grey line, secondary y-axis), and dark adapted b-wave (yellow line, secondary y-axis). Data from two typical CLN5$^{-/-}$ and CLN6$^{-/-}$ animals.

2.2.2 Comparison of maze traverse times and intracranial volume for control and affected cohorts

The ICV was compared to maze traverse times (Figure 2-10), in untreated animals of both genotypes. The cranial volume declined earlier than the maze traverse times. In both the CLN5 and CLN6 genotypes, the ICV were starting to decline at around 6 months of age, well before there was a slowing in maze traverse times.

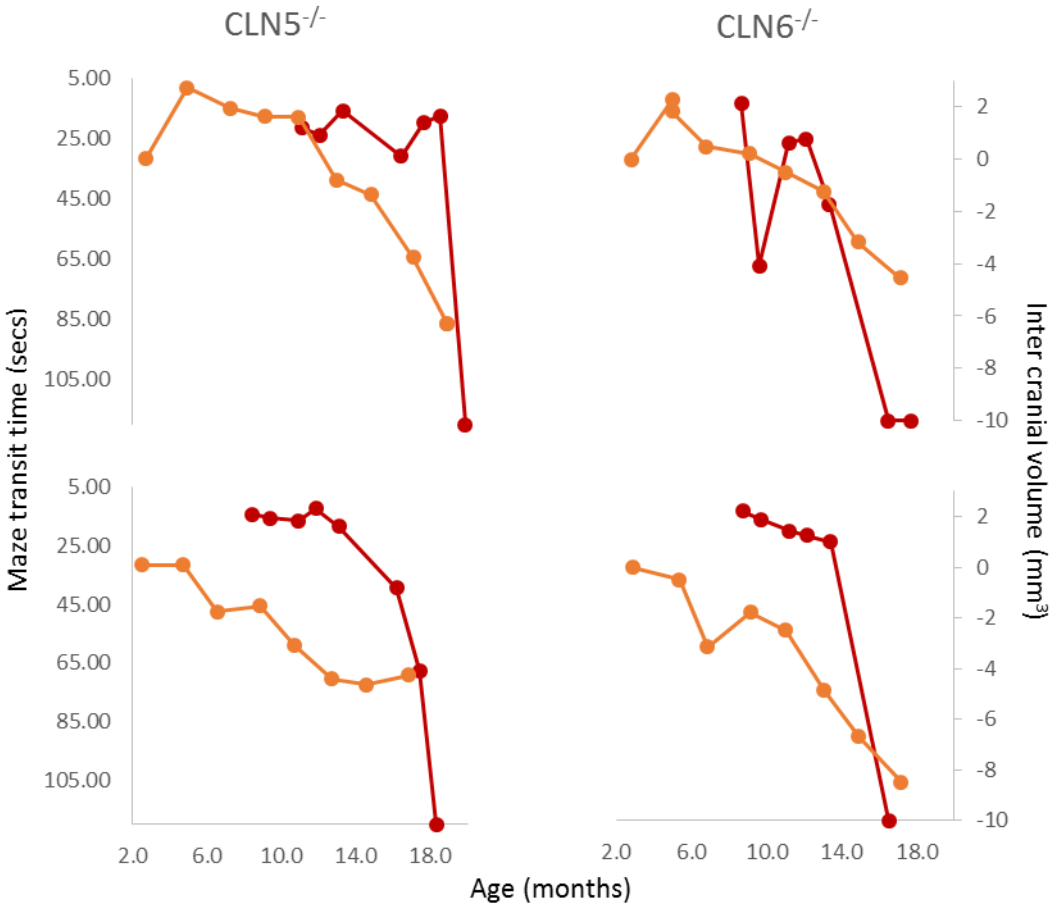

Figure 2-10. Representative data from two CLN5$^{-/-}$ and two CLN6$^{-/-}$ animals, showing maze traverse times (red line, primary y-axis), and intracranial volume change (orange line, secondary y-axis).

2.2.3 Comparison of traverse times for the CLN5$^{-/-}$ treated cohort

Maze traverse times for the four CLN5$^{-/-}$ animals who received brain-directed scAAV9.CLN5 gene therapy at 7 months were plotted against the mean normal and untreated CLN5$^{-/-}$ sheep data. Results are presented in Figure 2-11 and Table 2-3.

The treated and untreated CLN5$^{-/-}$ cohorts did not become significantly (95% c.i.) slower than their normal counterparts until 14 months of age (Table 2-3). One of the four treated animals (165) followed a similar trajectory in maze function to the untreated CLN5$^{-/-}$ sheep, failing to traverse at 19.4 months. The remaining three treated animals kept traversing until 22.9 months (1164) and 24.1 months (1172 and 1170).

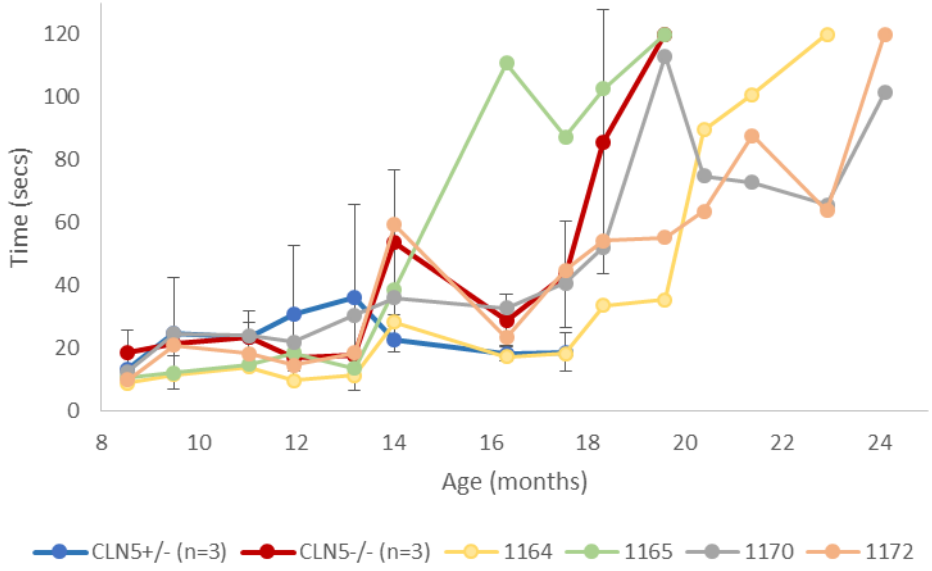

Figure 2-11. Mean (+/- sem) maze traverse times for normal and affected CLN5 cohorts, with times for four individual CLN5$^{-/-}$ animals treated by gene therapy at 7 months of age.

	Age (months)													
Tag#	8.5	9.5	11	12	13.2	14	16.3	17.5	18.3	19.6	20.4	21.4	22.9	24.1
Untreated CLN5$^{-/-}$														
1122	14.4	15.8	16.6	12.5	18.4	16.5	39.5	67.6	120					
1110	30.3	27.7	29.2	14.7	19.8	74.2	16.7	43.7	120					
1109	11.8	21.7	24.9	23.7	15.7	71	30.8	19.8	17.3	120				
Treated at 7 months of age CLN5$^{-/-}$														
1164	9	11.7	14	9.9	11.4	28.4	17.4	18.3	33.7	35.56	89.54	100.8	120	
1165	11	12.2	15	18.7	13.6	38.8	110.8	87.4	102.6	120				
1170	13	24.7	24.1	22.1	30.5	36.2	32.9	40.9	52.3	113	74.8	72.9	65.6	101.4
1172	10	21	18.3	14.7	18.6	59.4	23.6	45	54.3	55.4	63.6	87.6	63.8	

Table 2-3.Mean traverse times for individual untreated and treated CLN5$^{-/-}$ animals. Red figures indicate significantly slower (95% c.i.) than the normal control group (control group data ends at 17.5 months).

2.2.4 Comparison of maze distance ratios for the CLN5$^{-/-}$ treated cohort

The ratio of distance travelled to linear distance for the CLN5$^{-/-}$ animals who received brain-directed scAAV9.CLN5 gene therapy at 7 months was plotted against the mean normal and untreated CLN5$^{-/-}$ sheep data (Figure 2-12 and Table 2-4). The distance ratio of untreated animals was not significantly different ($p<0.05$) to normal controls up to 14 months of age. By 14 months of age all untreated and treated animals were taking a significantly ($P<0.05$) longer route

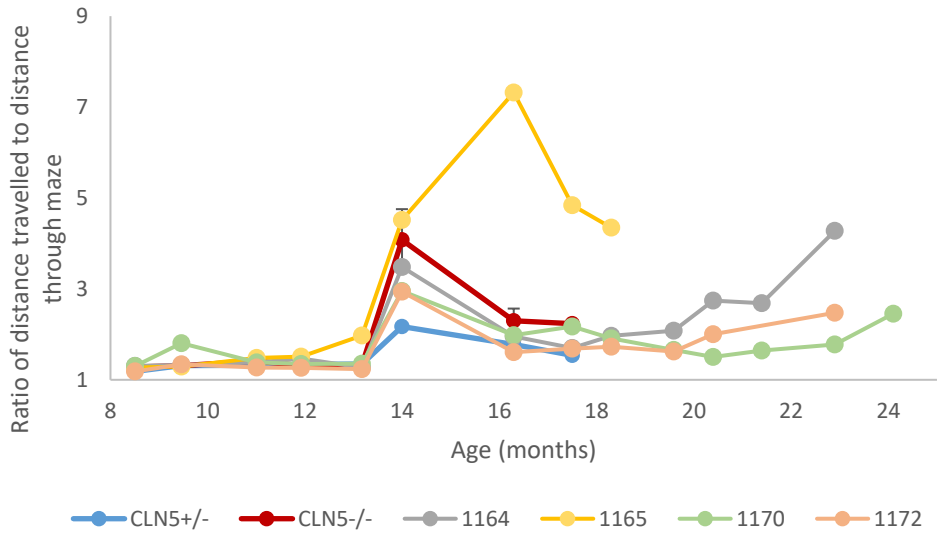

Figure 2-12. Mean (+/- sem) ratios of distance travelled to linear distance through the maze for normal and affected CLN5 cohorts, with ratios for four individual CLN5$^{-/-}$ animals treated by gene therapy at 7 months of age.

through the maze. Individual variations were apparent. Treated animal 1165 did not respond as robustly to the treatment. It was significantly slower (95% c.i.) than normal animals from 11 months and failed to complete the maze at 19.6 months. In comparison , treated animals 1164 and 1172 were not significantly ($P<0.05$) different from normal controls when last tested at 17.5 months of age, but gradually deteriorated and lost the ability to traverse at 22.9 months. Animal 1170 was also taking a much longer route, but was still able to traverse at 24.1 months.

	Age (months)													
Tag#	8.5	9.5	11	12	13.2	14	16.3	17.5	18.3	19.6	20.4	21.4	22.9	24.1
Untreated CLN5$^{-/-}$														
1122	1.32	1.30	1.31	1.32	1.39	3.63	2.24	3.32	F					
1110	1.30	1.30	1.39	1.31	1.29	3.30	1.48	1.78	F					
1109	1.22	1.34	1.52	1.30	1.29	5.30	3.17	1.60	1.52	F				
Treated at 7 months of age CLN5$^{-/-}$														
1164	1.31	1.33	1.38	1.47	1.27	3.48	1.95	1.70	1.97	2.08	2.74	2.68	4.28	
1165	1.27	1.29	1.48	1.51	1.98	4.51	7.32	4.84	4.35	F				
1170	1.3	1.80	1.38	1.35	1.36	2.95	1.98	2.17	1.91	1.66	1.50	1.64	1.77	2.45
1172	1.18	1.34	1.27	1.27	1.24	2.94	1.61	1.69	1.73	1.62	2.00	N/D	2.48	F

Table 2-4. Mean individual ratios of distance travelled to linear distance through the maze, for affected and CLN5$^{-/-}$ treated by gene therapy at 7 months of age. Red figures indicate significantly slower (95% c.i.) than normal control group (control group data ends at 17.5 months).

2.2.5 Comparison of maze and ERG findings for the CLN5$^{-/-}$ treated cohort

ERG recordings were being taken every two months on these normal, untreated and treated CLN5 animals as part of concurrent trails. Russell (K. Russell, 2017) had demonstrated the diminution of dark adapted a and b wave amplitudes in untreated CLN5$^{-/-}$ sheep over time. By 18 months their ERG traces were largely flat line. The treated sheep also lost their ERG responses, but this was delayed in most. In this trial the poorest performing treated animal (1165) (Figure 2-13), was also the first to lose its ERG response, and by 16 months it was a flat line. The two animals that were the best at traversing the maze at 22.9 months, also that had the best ERG responses (Figure 2-13) at 22 months.

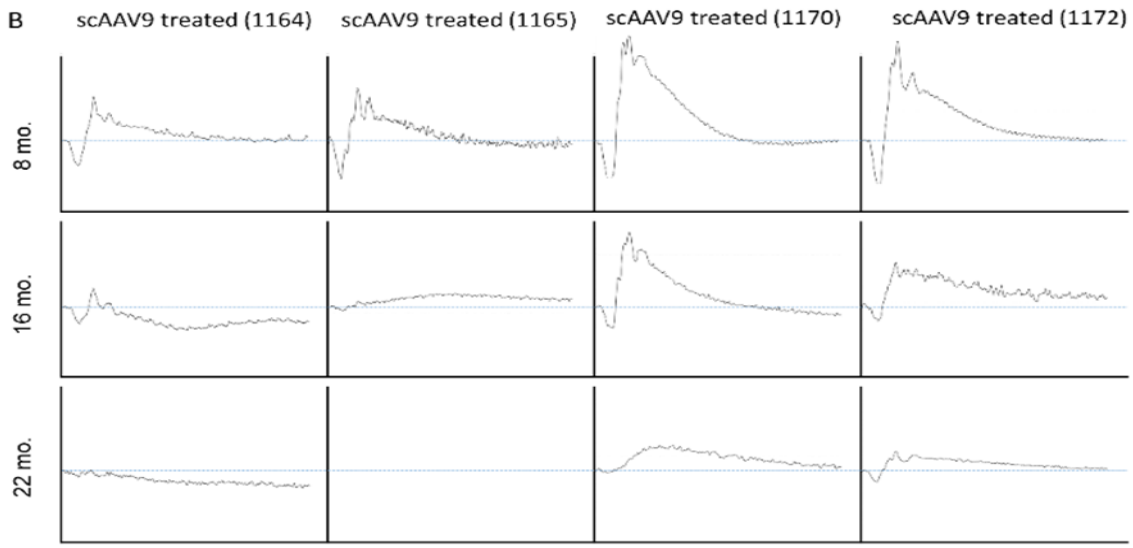

Figure 2-13. ERG responses of the four treated CLN5$^{-/-}$ sheep at 8, 16 and 22 months.

A summary of the clinical data, ERG responses and intracranial volume changes recorded in the concurrent study on the CLN5$^{-/-}$ untreated and treated sheep is presented in Table 2-5.

Animal	Genotype	Treatment	Failure in maze	Minimal ERG	ICV changes over time	Clinical outcome
1122	CLN5-/-	None	18.3	17.8	-10.5 mL (3 – 18 m)	Euthanised at 18.3 m, blind, advanced disease
1109	CLN5-/-	None	19.6	18	-12.9 mL (3 – 23 m)	Euthanised at 23.1m, blind, advanced disease
1110	CLN5-/-	None	18.3	18	-10.9 mL (3 – 19 m)	Euthanised at 19.6m, blind, advanced disease
1164	CLN5-/-	scAAV9.CLN5 at 7m	22.9	20.2	-1.2 mL (7 – 44 m)	Alive at 44 m, blind, mild clinical disease
1165	CLN5-/-	scAAV9.CLN5 at 7m	19.4	14.2	-4.2 mL (7 – 22m)	Euthanised at 22.5 m, blind, moderate clinical disease
1170	CLN5-/-	scAAV9.CLN5 at 7m	> 24.1	22.3	+2.4 mL (7 – 42 m)	Euthanised at 42.4 m, blind, moderate clinical disease
1172	CLN5-/-	scAAV9.CLN5 at 7m	22.9	20.5	-0.6 mL (7 – 22 m)	Euthanised at 22.3 m, visual deficits, mild clinical disease

Table 2-5. Summary of data collected on untreated and treated CLN5-/- animlas. NB. 1172 was euthanised as a comparative neuropathological control for 1165, not because of its clinical status.

The untreated CLN5$^{-/-}$ animal (1109) that was able to traverse the maze for longest was also the animal that survived the longest. The treated animal (1165) that failed to traverse the maze the first was also the animal that lost the most ICV volume and the first of the treated cohort to lose an ERG response. The only treated animal that failed to lose any ICV volume (1170) was the animal that was able to traverse the maze the longest.

2.2.6 Comparison of traverse times for the CLN6$^{-/-}$ treated cohort

Maze traverse times for five CLN6$^{-/-}$ animals who received brain-directed scAAV9.CLN5 or CLN6 gene therapy at 3 months were plotted against the normal and untreated CLN6$^{-/-}$ sheep data. Results are presented in Figure 2-14 and Table 2-6.

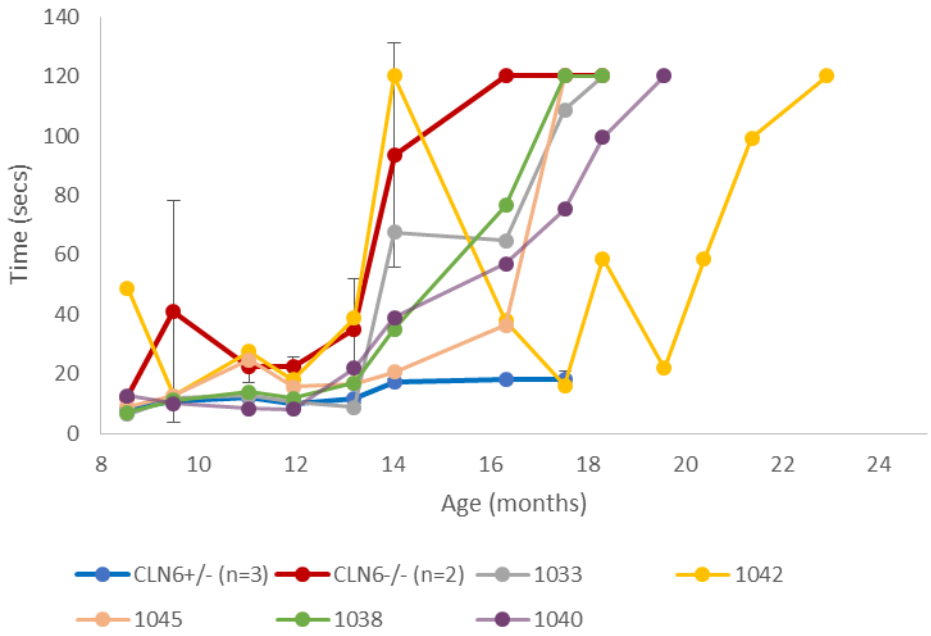

Figure 2-14Mean (+/- sem) maze traverse times for normal and affected CLN6 cohorts, with times for five individual CLN6$^{-/-}$ animals treated by gene therapy at 3 months of age.

The two animals in the untreated CLN6$^{-/-}$ cohort were significantly ($P<0.05$) slower than the untreated controls from when first tested at 8.5 months, and for the duration of the experiment (Table 2-6). The traverse times of the five CLN6$^{-/-}$ animals that had undergone gene therapy also slowed, but in general their loss of ability was delayed compared to the untreated CLN6$^{-/-}$ sheep (Figure 2-14). The treated CLN6$^{-/-}$ animals were all able to traverse the maze for longer than the

untreated affected cohort. Animal 1042 proved to be particularly capable, being able to traverse the maze until 22.9 months of age.

	Age (months)													
	8.5	9.5	11	12	13.2	14	16.3	17.5	18.3	19.6	20.4	21.4	22.9	24.1
Tag#	Untreated CLN6$^{-/-}$													
1008	12.8	67.5	25.9	24.7	46.8	120								
1014	11.9	14.8	18.8	20.5	22.8	66.8	120							
	Treated CLN6$^{-/-}$													
1033	6.5	11.9	12.7	10.5	8.7	67.6	64.7	108	120					
1038	7.2	11.3	14.1	11.7	17.1	35.1	76.6	120						
1040	12.6	10.0	8.4	8.1	21.8	39.0	57.1	75.4	99.4	120				
1042	48.8	12.8	27.5	18.0	38.8	120	37.9	16.2	58.7	22.0	58.5	99.2	120	
1045	9.0	12.8	24.7	15.8	16.8	20.8	36.5	120						

Table 2-6. Mean traverse times for individual untreated and treated CLN6$^{-/-}$ animals. Red figures indicate significantly slower (95% c.i.) than normal control group (control group data ends at 17.5 months).

2.2.7 Comparison of maze distance ratios for the CLN6$^{-/-}$ treated cohort

The ratio of distance travelled to linear distance for the CLN6$^{-/-}$ animals who received brain-directed scAAV9.CLN5 or CLN6 gene therapy at 3 months was plotted against the CLN6$^{-/-}$ untreated and CLN6$^{+/-}$ normal controls (Figure 2-15 and Table 2-7). The distance travelled ratio showed all treated and untreated CLN6$^{-/-}$ animals taking a significantly (95% c.i.) longer route through the maze at 14 months of age. One of the affected controls (1008) travelled significantly (95% c.i.) further from 12 months of age, and failed to negotiate the maze at all at 14 months. Prior to that age there had been individual animals taking significantly (95% c.i.) longer routes at isolated time points. The distance ratio data showed that treated animal 1042 followed a more direct route, similar to normal controls (Table 2-7), for most of the study, until failing to traverse at 22.9 months of age.

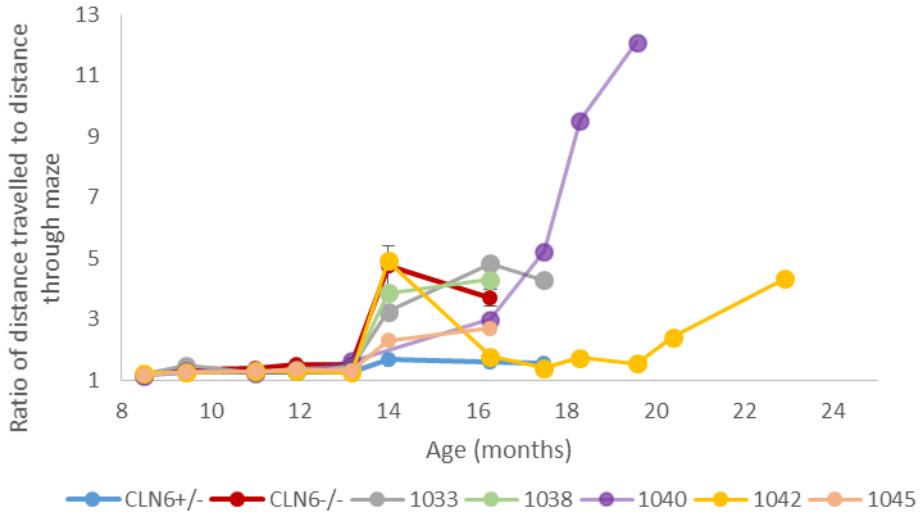

Figure 2-15. Mean (+/- sem) ratios of distance travelled to linear distance through the maze for normal and affected CLN6 cohorts, with ratios for five individual CLN6$^{-/-}$ animals treated by gene therapy at 3 months of age.

	Age (months)													
	8.5	9.5	11	12	13.2	14	16.3	17.5	18.3	19.6	20.4	21.4	22.9	24.1
Tag#	Untreated CLN6$^{-/-}$													
1008	1.17	1.34	1.35	1.69	1.57	F								
1014	1.16	1.33	1.46	1.35	1.44	6.10	F							
	Treated CLN6$^{-/-}$													
1033	1.20	1.49	1.24	1.36	1.44	3.25	4.83	4.29	F					
1038	1.20	1.27	1.30	1.30	1.38	3.88	4.30	F						
1040	1.16	1.31	1.25	1.29	1.63	ND	3.00	5.24	9.53	F				
1042	1.25	1.25	1.29	1.31	1.26	4.93	1.78	1.40	1.75	1.55	2.41	ND	F	
1045	1.18	1.27	1.30	1.35	1.32	2.30	2.71	F						

Table 2-7. Mean individual ratios of distance travelled to linear distance through the maze, for affected and CLN6$^{-/-}$ sheep treated by gene therapy at 3 months of age. Red figures indicate significantly slower (95% c.i.) than normal control group (control group data ends at 17.5 months of age). ND = No data.

2.3 Discussion

This study established that normal control and CLN5 and CLN6 affected sheep were able to navigate a long and complex maze. Sheep were enrolled at 8.5 months of age and several iterations of the maze were tested. The final version of the maze was not arrived at until the animals were 16 months of age but by then the maze had been developed into a quick and simple format that could be operated by a single person. Perhaps due to continued changes in the maze during its development, maze testing was not able to distinguish between healthy normal and affected animals at an earlier age than our other *in vivo* measures however it was a useful adjunct method to assess efficacy after CLN5 or CLN6 gene therapy. This study showed that the ability of affected animals to traverse the maze decreased as Batten disease progressed, and the gene therapy treatments allowed the animals to traverse for longer. However although a good separation in maze traverse performance was achieved between the affected and normal cohorts, this also coincided with the severe deterioration of the eyesight of the affected and treated animals. This makes it difficult to fully attribute this loss of ability to traverse the maze to cognitive function.

Time to traverse the maze was found to be very variable and not necessarily a good indicator of a sheep's ability to traverse the maze. The two breeds of sheep behave very differently in the maze. The South Hampshires (CLN6) are of a more nervous disposition, and seem to acclimate to human contact slower than the Borderdales (CLN5). Hence the desire to get away from the operator, and through the maze appears to be more powerful in the South Hampshire as opposed to the Borderdales. The South Hampshire sheep also seems to have a stronger flocking instinct, so the attraction of flock-mates at the end of the maze is stronger. As an example at 13.2 months the fastest Borderdale transited in 8.9seconds the slowest in 84.3 seconds which indicates the variability in fear of the operator and desire to return to conspecifics. Habituation to human contact has been described before in this type of testing scenario (Erhard, Elston, &

Davidson, 2006), and other researchers have also commented on the fact that Borderdale sheep would stop during passage through their maze test (McBride et al., 2016). Hence maze traverse time appears to be a good indicator of performance for the South Hampshire animals but not Borderdales.

The use of the high precision GPS unit to calculate the path length through the maze significantly reduced the variability in the CLN5 Borderdale data, and allowed analyses such that animals stopping and standing did not influence the results. Thus distance ratios are a better indicator of performance for Borderdale sheep.

Small numbers in cohorts, and high individual variability meant that statistical significance was often difficult to show, but at an individual level the maze results correlated well with other clinical observations. Of note, maze data showed a differential response to post-symptomatic scAAV9.CLN5 treatment. Three of the animals (1164, 1170 and 1172) responded well to treatment, they had stabilised brain volumes and were able to negotiate the maze up to 20.2, 24.1, and 22.9 months respectively. ERG measurements would indicate that all three animals had very limited eyesight at 23 months of age. In contrast, treated animal 1165 lost retinal function at 14.2 months, failed to traverse the maze at 19.4 months of age and also lost the most intracranial volume over its lifetime.

The treated CLN6 animals also had varied responses to treatment. They performed better than the untreated CLN6$^{-/-}$ controls, but apart from one animal (1042) they did not respond as well as the CLN5 genotype. The CLN6 treated animals were euthanised with moderate or advanced disease between 18.2 and 22.3, in contrast to the CLN5 treated animals who were euthanised between 22.3 and >44 months. The CLN6 treated animals also lost more ICV volume than their CLN5 counterparts, between -5.9 and -10.6ml, versus +2.4ml to -4.2ml.

The maze data collected correlated well with the ICV volume loss, time to euthanasia, and ERG data collected on the sheep of both CLN5 and CLN6 genotypes. The treated animals would ultimately fail the maze, due to deteriorating eyesight. The treatments given were specifically brain directed and, whilst may protect against atrophy of the visual cortex of the brain, would not ameliorate the deterioration of the retina of the eye which confers blindness at around 12-16 months of age. Thus loss of vision becomes a confounding factor, when using a visual based maze to gauge cognitive decline. This study intended to assess the suitability of a maze as an early measure of disease progression, before the loss of eyesight. Due to the length of time taken to arrive at the final conformation of the maze, it was decided to repeat the experiment, using the final more rigorous form of the maze developed in this study, in a younger cohort of animals. This additional study would investigate whether the final maze configuration was able to discriminate between a normal and affected animal at an earlier time than was achieved in this study.

3 Experiment 2

3.1 Introduction

Experiment 1 developed a more stringent maze that could distinguish between normal and affected animals CLN5$^{-/-}$ at 14 months and between normal and affected CLN6$^{-/-}$ at 11 months of age. This study used that final version of the maze at a younger starting point of 5.2 months of age. The hypothesis was that the more complex maze would allow earlier discrimination between normal and affected animals, and hence allow earlier monitoring of treatment efficacy. This study also introduced maze reversal learning as a comparatively more demanding task. This has been demonstrated in previous sheep maze studies (Hunter et al., 2015; Johnson et al., 2012; Morton & Avanzo, 2011). Morton and Avanzo used coloured buckets as visual stimuli, with one colour consistently contained the food reward, and all 7 animals reached an 80% correct choice criterion after 56 choices. When the colour of the bucket the reward was contained in was reversed the 80% criterion was reached by all animals after 88 choices. The study by Johnson et al (2012) was based on spatial learning, and exposed sheep (n=12) to a T maze with a feed reward consistently on one side. After 9 exposures to this maze, all animals went to the correct side. The side of the food reward was then switched to the alternative side of the maze, and after 9 exposures to the change, 78% of animals went directly to the new site of the food reward. Therefore the capability of sheep to learn a reversal in stimuli or a spatial reversal has been established, and in this study the reversal was spatial, but reinforced with a learned visual stimuli.

3.2 Materials and methods

3.2.1 Animals and experimental design

This study was to evaluate the final configuration of the maze used in experiment 1, as a tool for assessing Batten disease progression. The study was carried out with $CLN5^{+/-}$ and $CLN6^{+/-}$ normal controls (n=3), and $CLN5^{-/-}$ and $CLN6^{-/-}$ affected controls (n=3). Three treated $CLN5^{-/-}$ cohorts were included; one treated by scAAV9.CLN5 at 3 months of age (n=6), another similarly treated at 6 months (n=3) and a third cohort treated at 9 months (n=3). The $CLN6^{-/-}$ treatment group was treated by either scAAV9.CLN5 (n=3), or a combination of scAAV9.CLN5 and CLN6 (n=3) at 3 months of age (Table 3-1).

The animals were first tested at a mean age of 5.2 months (min 4.9, max 5.5 months), and were subjected to five consecutive runs through the maze. When the animals were 9.8, 11.3, 12.4 and 14 months old they were additionally tested in a reverse maze. After the initial five runs the barrier gates were slid across to the opposite side and the maze was then reversed. The sheep were run through the reversed maze four times. Testing of all animals finished at 14 months old.

Sheep #	Genotype	Treatment	Other *in vivo* assessments
1002	CLN6$^{+/-}$	None, normal control	None
1004	CLN6$^{+/-}$		
1006	CLN6$^{+/-}$		
1100	CLN5$^{+/-}$		
1106	CLN5$^{+/-}$		
1116	CLN5$^{+/-}$		
1027	CLN6$^{-/-}$	None, affected control	CT, ERG
1046	CLN6$^{-/-}$		
1047	CLN6$^{-/-}$		
1119	CLN5$^{-/-}$		
1125	CLN5$^{-/-}$		
1142	CLN5$^{-/-}$		
1012	CLN6$^{-/-}$	sc AAV9 CLN6@ 3 months	CT. ERG
1020	CLN6$^{-/-}$		
1030	CLN6$^{-/-}$		
1014	CLN6$^{-/-}$	sc AAV9 CLN5 and CLN6 @ 3 months	
1023	CLN6$^{-/-}$		
1031	CLN6$^{-/-}$		
1102	CLN5$^{-/-}$	sc AAV9 CLN5@ 3 months	
1104	CLN5$^{-/-}$		
1111	CLN5$^{-/-}$		
1120	CLN5$^{-/-}$		
1123	CLN5$^{-/-}$		
1128	CLN5$^{-/-}$		
1185	CLN5$^{-/-}$	sc AAV9 CLN5 @ 6 months	CT. ERG
1186	CLN5$^{-/-}$		
1187	CLN5$^{-/-}$		
1143	CLN5$^{-/-}$	sc AAV9 CLN5 @ 9 months	
1163	CLN5$^{-/-}$		
1165	CLN5$^{-/-}$		

Table 3-1. List of animals used in Experiment 2.

3.2.2 GPS tracking

Animals were tracked with the same equipment, and in the same manner as described in Experiment 1.

3.2.3 Maze

The maze was the same as the final configuration of the maze described in Experiment 1.

3.2.4 Gene therapy

The gene therapy procedure is described in experiment 1. The cohorts for this experiment are shown in Table 3-1.

3.2.5 Statistical techniques

Means and corresponding SEM was calculated for each group at each time point. Student's t-tests were performed to test each group against the normal controls at each time point. Differences were regarded as significant where $P<0.05$. Where individual treated and untreated affected animals were compared to the control group, they were considered to be significantly different from controls when they were more than 1.96 standard deviations away from normal control mean values.

3.3 Results

3.3.1 Heterozygous normal control animals

Mean traverse times for heterozygous normal control sheep of each breed were compared (Figure 3-1). Times were only significantly different ($P<0.05$) for the $CLN5^{+/-}$ and $CLN6^{+/-}$ control groups at 6.4 and 9.7 months, when the $CLN5^{+/-}$ were slower than the $CLN6^{+/-}$. The $CLN5^{+/-}$ and $CLN6^{+/-}$ cohorts were both significantly ($P<0.05$) slower on the first run through the maze than in all subsequent runs.

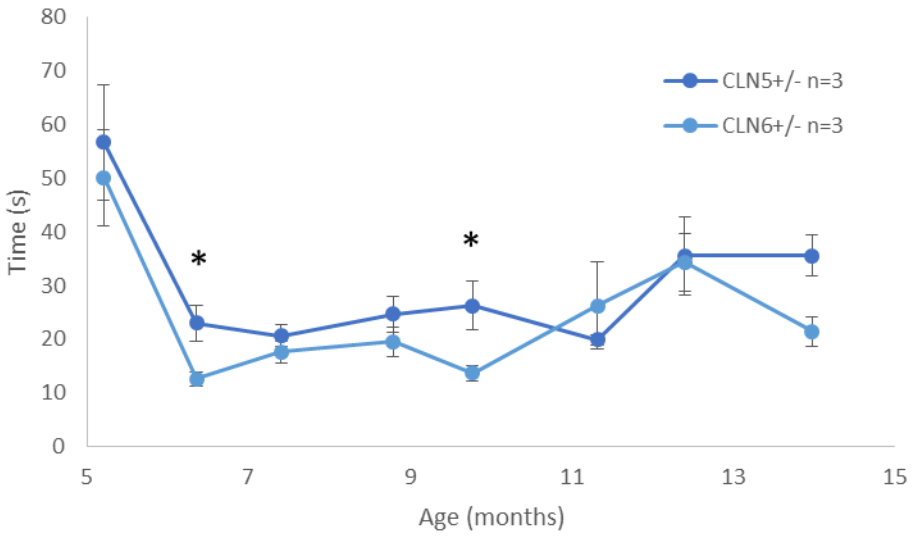

Figure 3-1. Mean (+/- sem) maze traverse times for normal CLN5$^{+/-}$ and CLN6$^{+/-}$ cohorts (n=3). Asterisk denotes significant difference (P<0.05).

The ratio of distance travelled to the linear distance through the maze for both cohorts of normal control sheep were also plotted (Figure 3-2). There was no significant difference (P>0.05) at any times although, as in the time measurement, both genotypes travelled significantly (P<0.05) longer distances on their first exposure to the maze.

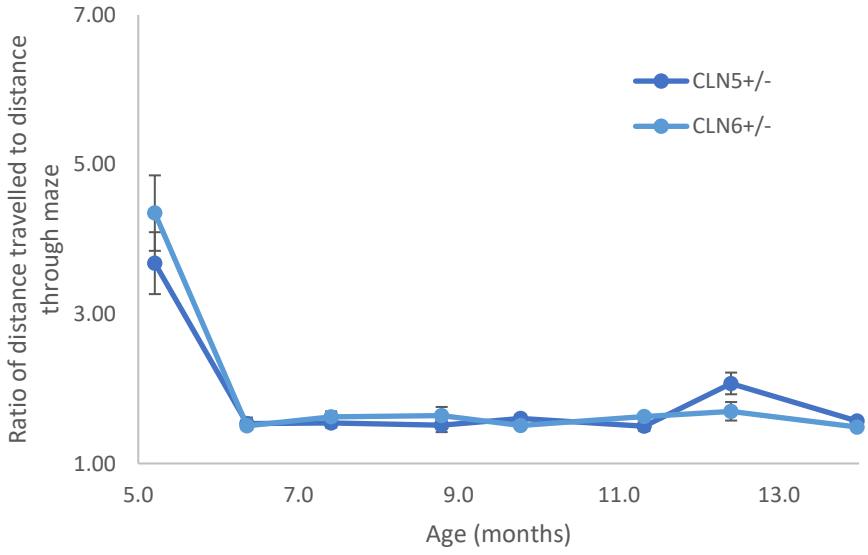

Figure 3-2. Mean (+/- sem) ratio of distance travelled to linear distance through the maze for normal CLN5$^{+/-}$ and CLN6$^{+/-}$ cohorts (n=3).

3.3.2 Maze transit times for normal control and affected cohorts

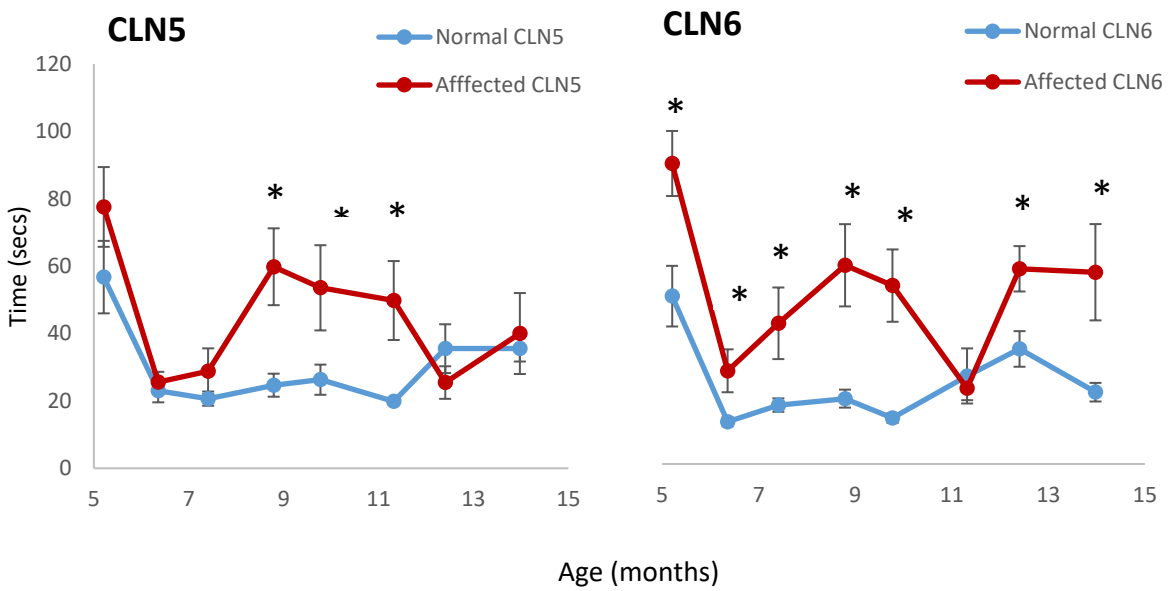

Figure 3-3. Mean (+/- sem) maze traverse times for normal (blue line) and affected (red line) CLN5 and CLN6 cohorts (n=3). Asterisk denote significant differences (P<0.05).

The mean traverse times for CLN5$^{-/-}$ affected and CLN5$^{+/-}$ normal animals (Figure 3-3) showed that the affected cohort was only significantly (P<0.05) slower at three time points (8.8, 10 and 11.3 months). The data for the CLN6 animals (Figure 3-3) showed that the CLN6$^{-/-}$ cohort was significantly (P<0.05) slower than the normal controls up until 11.3 months of age, and then again at 12 and 14 months of ages.

3.3.3 Maze distance ratios for normal control and affected cohorts

The distance travelled as a proportion of the linear distance was calculated for all four cohorts (Figure 3-4) and compared to the time results (Figure 3-3).There was much less variability in the distance data. The CLN5$^{-/-}$ affected sheep took a significantly (P<0.05) longer path than their normal CLN5$^{+/-}$ counterparts at most of the time points, and the mean distance covered by the normal cohort was always less than the affected cohort took. The CLN6$^{-/-}$ sheep took a significantly (P<0.05) longer path than the normal CLN6$^{+/-}$ sheep at the two earliest time points of 5.2 and 7.4 months, but for the rest of the experiment there was no significant (P>0.05) difference between the cohorts.

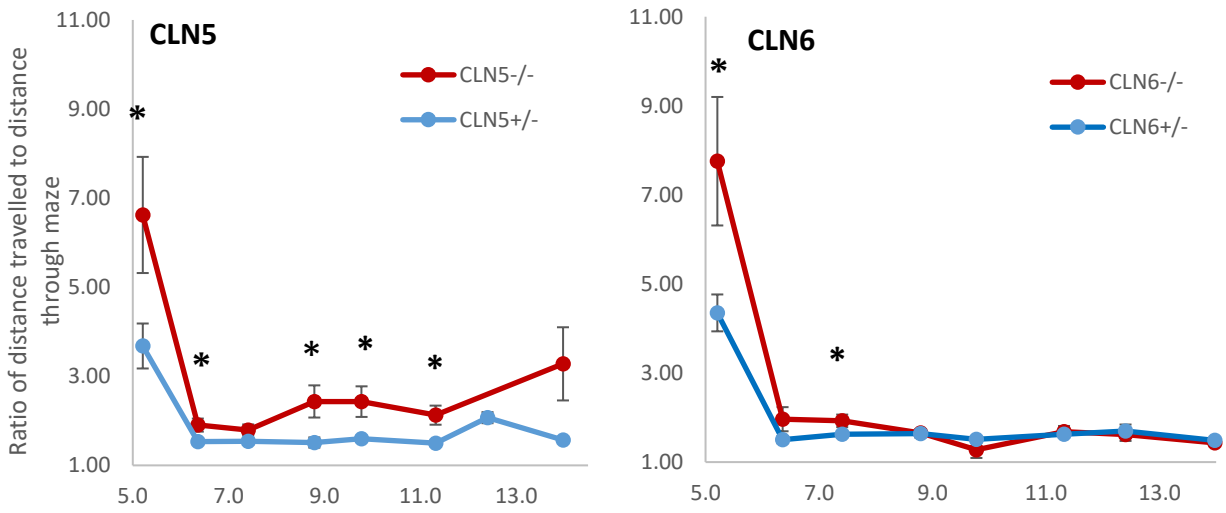

Figure 3-4. Mean (+/- sem) ratios of distance travelled to linear distance through the maze for CLN5 and CLN6 normal (blue line) and affected (red line) cohorts (n=3). Asterisks denote significant differences (P<0.05).

3.3.4 Maze transit times for the CLN5$^{-/-}$ treated cohort

Maze transit times for the three CLN5$^{-/-}$ cohorts that received sc AAV9 CLN5 treatment at 3, 6 and 9 months of age were plotted against the normal and untreated CLN5$^{-/-}$ sheep data. Results are presented in *Figure 3-5* and Table 3-2.

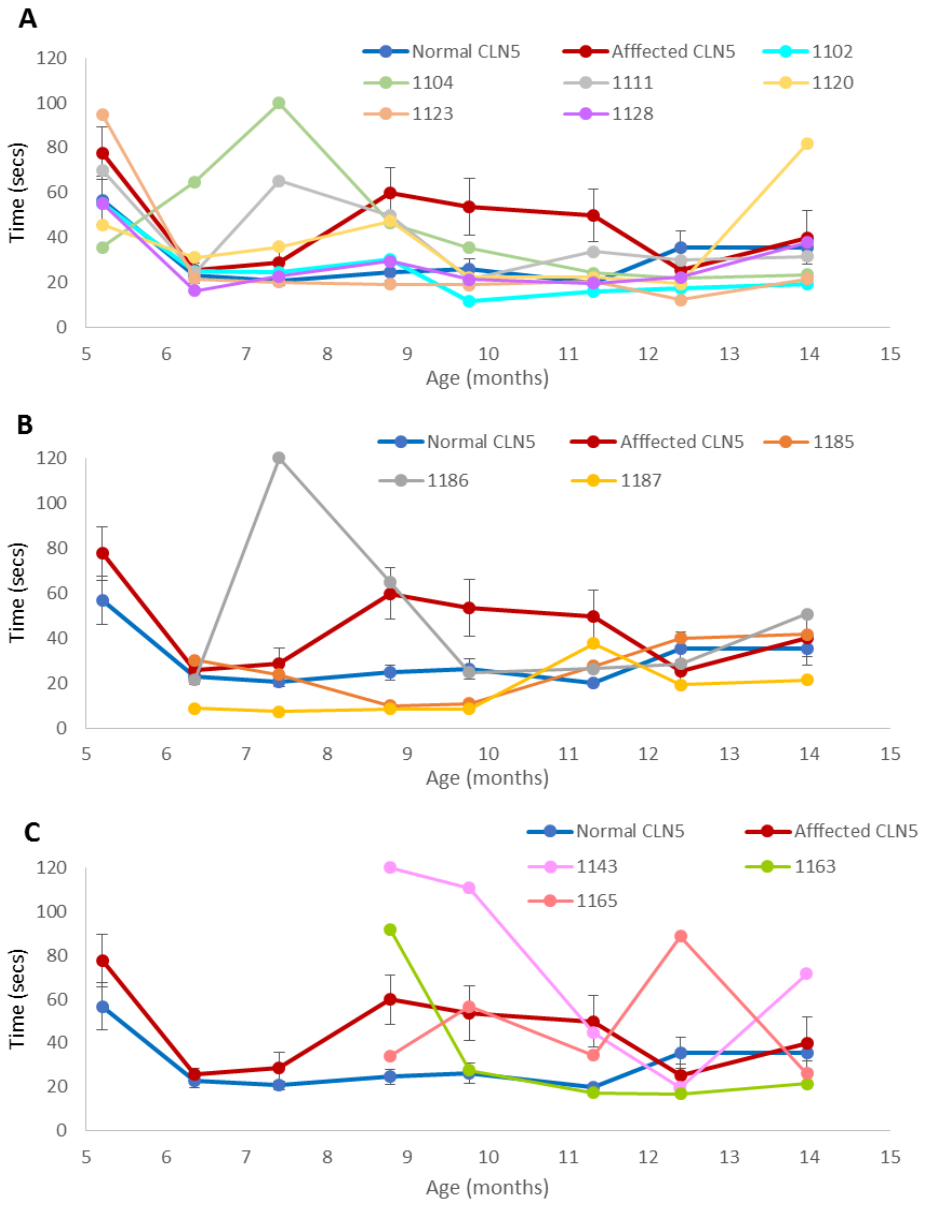

Figure 3-5. Maze traverse times for individual CLN5$^{-/-}$ sheep treated by gene therapy at 3 months (A), 6 months (B) or 9 months (C), plotted against mean times for normal (blue line) and affected (red line) controls.

Sheep treated at 3 and 6 months traversed the maze in similar or faster times than the untreated affected controls at most time points. Treatment at 9 months resulted in more erratic traverse times. Two of the three animals treated at 9 months of age were typically slower than untreated affected sheep. However one 9-month treated animal (1163) was consistently as fast as normal controls at all times after its initial baseline run. Individual data for the untreated and treated CLN5$^{-/-}$ animals (Table 3-2) showed a random scattering of times that were significantly slower

(95% c.i.) than the normal control cohort throughout the course of the experiment. There was no trend of either the untreated or treated animals becoming significantly slower at the end of the 14 months.

	Age (months)							
	5.2	6.4	7.4	8.8	9.8	11.3	12.4	14.0
affected $CLN5^{-/-}$								
1119	44.1	20.3	38.8	99.1	97.6	104.1	34.4	15.1
1125	77.4	15.9	22.9	61.8	45.4	29.7	21.1	28.9
1142	111.2	40.5	24.6	18.5	17.6	15.6	20.9	15.8
Treated $CLN5^{-/-}$ (3mth)								
1102	55.2	24.9	24.5	30.1	11.7	15.9	17.4	19.4
1104	35.6	64.8	99.9	46.3	35.6	24.3	21.8	23.6
1111	70.0	24.6	65.2	49.8	21.4	33.8	30.0	31.7
1120	45.8	31.1	35.9	47.4	22.4	22.3	19.5	81.8
1123	94.8	21.7	20.3	19.4	19.1	20.4	12.4	21.7
1128	55.3	16.5	22.8	29.6	21.3	19.7	22.4	37.9
Treated $CLN5^{-/-}$ (6mth)								
1185		30.2	23.7	9.8	10.9	27.5	40.0	41.7
1186		21.6	120.0	64.9	24.8	26.4	28.5	50.8
1187		8.8	7.3	8.4	8.4	37.6	19.2	21.3
Treated $CLN5^{-/-}$ (9mth)								
1143				120.0	110.7	44.8	19.9	71.5
1163				91.6	27.5	17.3	16.8	21.4
1165				34.1	56.6	34.4	88.6	26.2

Table 3-2 Mean traverse times for individual $CLN5^{-/-}$ affected and $CLN5^{-/-}$ treated by gene therapy at 3, 6 and 9 months of age. Red figures indicate significantly slower (95% c.i.) than normal control cohort

3.3.5 Maze distance ratios for the $CLN5^{-/-}$ treated cohort

As before the ratio of distance travelled to linear distance for the $CLN5^{-/-}$ animals who received brain-directed sc AAV9 CLN5 treatment at 3, 6 or 9 months was plotted against the mean normal and untreated $CLN5^{-/-}$ sheep data (Figure 3-6).

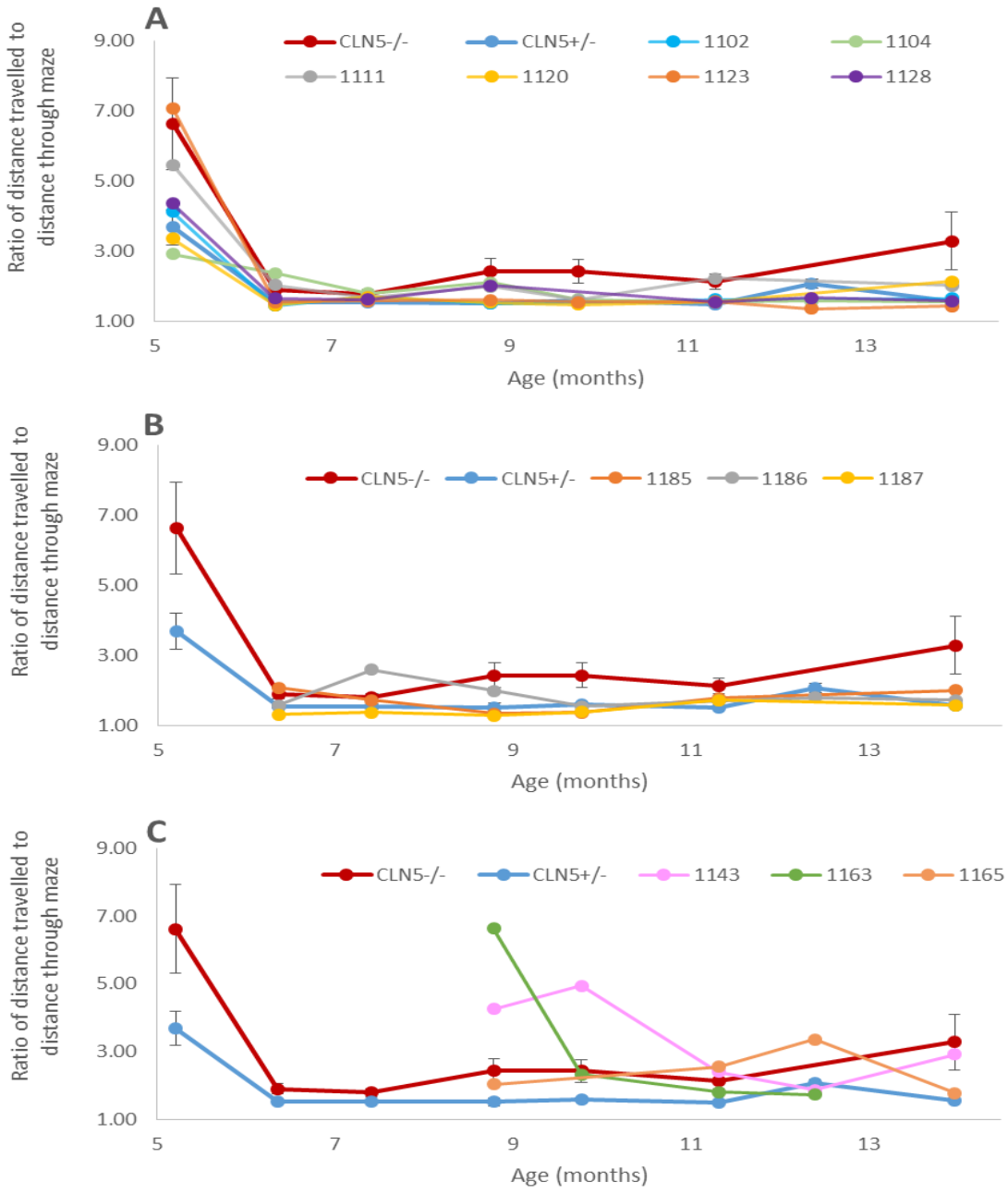

Figure 3-6. Ratios of distances travelled to the linear distance through the maze for individual CLN5$^{-/-}$ sheep treated by gene therapy at 3 months (A), 6 months (B) or 9 months (C), plotted against mean times for normal (blue line)and affected (red line) controls.

This showed that the apart from occasional individuals, the 3 and 6 month treated cohorts traversed the maze by a shorter route than the average untreated affected animals. Two of the three animals in the 9 month treated cohort frequently took a longer path then the affected control cohort, whilst animal (1163) was more direct.

	Age (months)							
	5.2	6.4	7.4	8.8	9.8	11.3	12.4	14.0
affected CLN5-/-								
1119	5.08	1.61	1.81	2.46	3.85	2.69	nd	3.05
1125	3.21	1.55	1.58	2.76	1.89	1.61	nd	1.65
1142	11.58	2.46	2.00	2.09	1.56	1.97	nd	5.61
Treated CLN5$^{-/-}$ (3mth)								
1102	4.12	1.45	1.71	1.51	1.53	1.62	nd	1.66
1104	2.92	2.37	1.79	2.11	1.62	1.56	1.58	1.55
1111	5.45	2.04	1.68	1.99	1.62	2.23	nd	2.02
1120	3.36	1.47	1.70	1.54	1.47	1.56	nd	2.13
1123	7.07	1.56	1.58	1.61	1.54	1.57	1.36	1.43
1128	4.35	1.65	1.62	2.03	nd	1.55	1.67	1.59
Treated CLN5-/- (6mth)								
1185		2.07	1.73	1.34	1.37	1.79	nd	2.00
1186		1.57	2.59	1.98	1.55	1.71	1.80	1.73
1187		1.31	1.37	1.29	1.39	1.72	nd	1.57
Treated CLN5-/- (9mth)								
1143				4.26	4.94	2.41	1.87	2.92
1163				6.64	2.32	1.81	1.73	nd
1165				2.03	nd	2.56	3.37	1.78

Table 3-3. Mean individual ratios of distance travelled to linear distance through the maze, for CLN5$^{-/-}$ affected and CLN5$^{-/-}$ treated by gene therapy at 3, 6 or 9 months of age. Red figures indicate significantly slower (95% c.i.) than normal control cohort. nd = no data, due to failure of GPS unit.

The individual data (Table 3-3) showed that the untreated CLN5$^{-/-}$ animals were frequently taking a longer path than the normal controls. Animals treated at 3 months rarely took a longer path, whilst the 6 month treated cohort began to take a longer path from 11.3 months. The 9 month treated sheep were nearly always taking a significantly ($P<0.05$) longer path than normal controls.

3.3.6 Maze transit times of the CLN6$^{-/-}$ treated cohort

Maze transit times for the CLN6$^{-/-}$ cohort that received sc AAV9 CLN5 or CLN6 treatment at 3 months of age were plotted against the normal and untreated CLN5$^{-/-}$ sheep data in Figure 3-7and Table 3-4. There was considerable variation in times from individuals within the untreated affected cohort. One animal (1027) was always significantly (95% c.i.) slower than the normal controls, and failed to transit the maze at 12.4 months. Another (1047) was only significantly slower on two occasions in the middle of the study (8.8 and 9.8 months), and the third (1046) always traversed the maze at times equivalent to its normal counterparts.

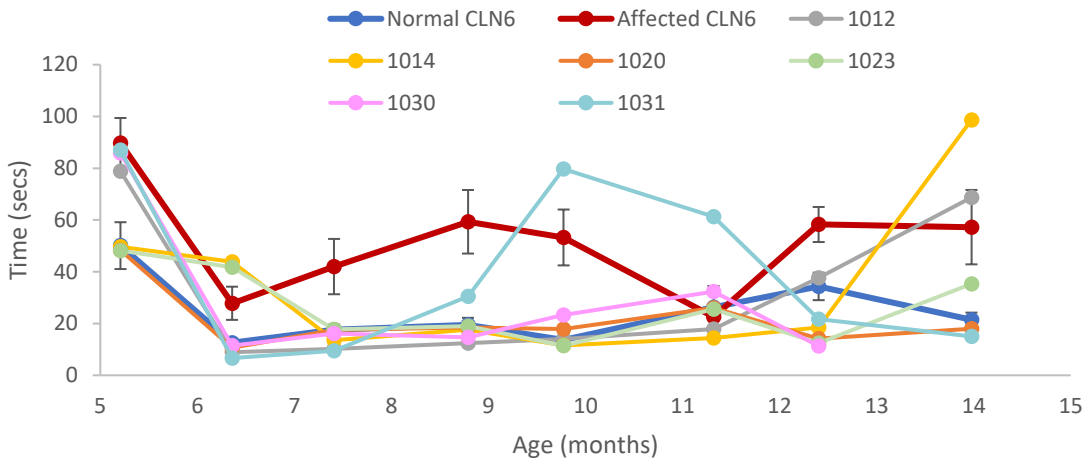

Figure 3-7. Maze traverse times for individual CLN6$^{-/-}$ sheep treated by gene therapy at 3 months, plotted against mean times for normal (blue line) and affected (red line) controls.

	Age (months)							
	5.2	6.4	7.4	8.8	9.8	11.3	12.4	14.0
	Affected CLN6$^{-/-}$							
1027	120	52.6	81.4	89.7	89.8	33.9	120	
1046	55.8	20.1	25.6	17.1	16.8	21.6	16.4	25.7
1047	93.2	10.8	19.0	71.1	53.1	12.2	38.4	13.7
	Treated CLN6$^{-/-}$ (3mth)							
1012	78.9	8.9	10.2	12.4	14.1	17.9	37.6	68.7
1014	49.7	43.8	13.6	17.6	11.5	14.4	18.5	98.7
1020	48.3	10.7	17.5	18.5	17.8	26.0	14.1	18.0
1023	48.2	41.7	17.7	19.0	11.4	25.5	12.4	35.3
1030	85.9	11.5	16.1	14.6	23.3	32.3	11.3	nd
1031	87.0	6.7	9.5	30.5	79.8	61.3	21.7	15.0

Table 3-4. Mean traverse times for individual CLN6$^{-/-}$ affected and CLN6$^{-/-}$ animals treated by gene therapy at 3 months of age. Red figures indicate significantly slower (95% c.i.) than normal control cohort. nd = no data.

There were very few occurrences over the course of the experiment of the treated animals being significantly (95% c.i.) slower than the normal cohort.

3.3.7 Maze distance ratio of the CLN6$^{-/-}$ treated cohort

The ratio of distance travelled to linear distance for the CLN6$^{-/-}$ animals who received brain-directed gene therapy at three months were plotted against the CLN6$^{-/-}$ affected and CLN6$^{+/-}$ normal controls (Figure 3-8). The individual data (Table 3-5) show that only one of the untreated

controls (1027) took a significantly (95% c.i.) longer route than a normal animal. Treated animals travelled similar distances to the normal controls until towards the end of the experiment and at the last testing at 14 months four of the five treated animals travelled a significantly (95% c.i.) longer path through the maze.

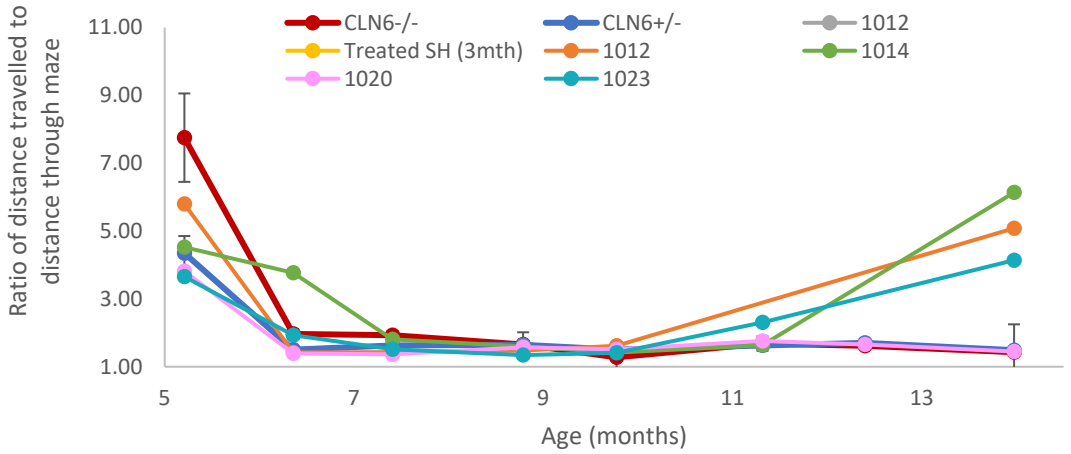

Figure 3-8. Ratio of distance travelled to linear distance through the maze for individual CLN6$^{-/-}$ sheep treated by gene therapy at 3 months of age, plotted against mean times for normal (blue line) and affected (red line) controls.

	Age (months							
	5.2	6.4	7.4	8.8	9.8	11.3	12.4	14.0
Affected CLN6$^{-/-}$								
1027	12.75	3.05	2.20	1.68	nd	2.10	nd	F
1046	3.61	1.40	nd	1.46	nd	1.47	1.62	1.37
1047	6.90	1.50	1.58	1.87	1.28	1.48	nd	1.53
Treated CLN6$^{-/-}$ (3 months)								
1012	5.80	1.42	1.43	1.47	1.62	nd	nd	5.09
1014	4.53	3.77	1.81	1.60	1.42	1.64	nd	6.14
1020	3.81	1.39	1.37	1.57	1.51	1.76	1.65	1.45
1023	3.66	1.93	1.51	1.34	1.41	2.30	nd	4.14
1030	6.04	1.72	1.37	1.85	1.65	nd	1.60	nd
1031	6.05	1.28	1.42	1.49	2.08	1.90	1.74	1.67

Table 3-5. . Mean individual ratios of distance travelled to linear distance through the maze, for CLN6$^{-/-}$affected and CLN6$^{-/-}$ animals treated by gene therapy at 3 months of age. Red figures indicate significantly slower (95% c.i.) than normal control cohort. nd = no data, due to failure of the GPS unit.

3.3.8 Reverse maze traverse times of normal and affected control cohorts

There were no significant differences (P<0.05) in the times taken by the normal CLN5$^{+/-}$ and CLN6$^{+/-}$ cohorts at any time (data not shown). The normal and affected cohorts of both breeds were plotted against each other (Figure 3-9). Their ability to traverse the reversed maze was very variable, and the individual data (Table 3-6) show that over the time they were tested there was no tendency for the affected cohort to become slower as the disease progressed.

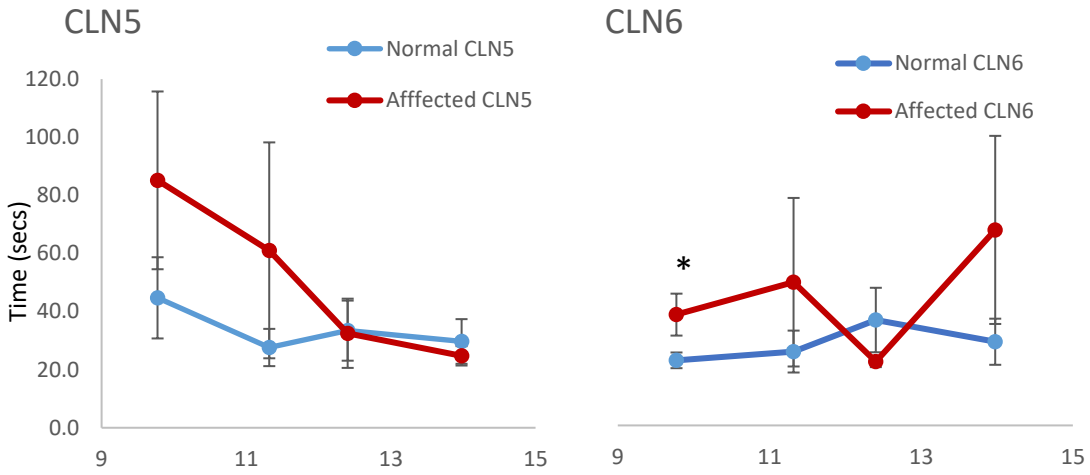

Figure 3-9. Mean (+/- sem) reverse maze traverse times for CLN5 and CLN6 normal (blue line) and affected (red line) cohorts. Asterisks denote significant difference (P<0.05).

	Age (months)			
	9.8	11.3	12.4	14.0
CLN5$^{-/-}$ affected				
1119	120	120	51.7	28.2
1125	98.8	44.1	20.3	26.7
1142	36.7	19.1	25.6	19.4
CLN6$^{-/-}$ affected				
1027	47.3	96.7	nd	120
1046	27.1	20.0	20.2	33.5
1047	40.7	32.0	24.0	49.5

Table 3-6. Reverse maze traverse times for individual CLN5$^{-/-}$ and CLN6$^{-/-}$ affected animals. Red figures indicate significantly (95% c.i.) slower than normal control cohort. nd = no data.

3.3.9 Reverse maze distance ratios of normal and affected control cohorts

The distance travelled as a proportion of the linear distance was calculated for all four cohorts, and the affected means plotted against the means of the normal controls (Figure 3-10). The CLN5 affected cohort took a significantly (P<0.05) longer path through the maze at 11.3 and 14 months of age. Overall there was a trend for both CLN5 genotypes to reduce path length through the maze over time.

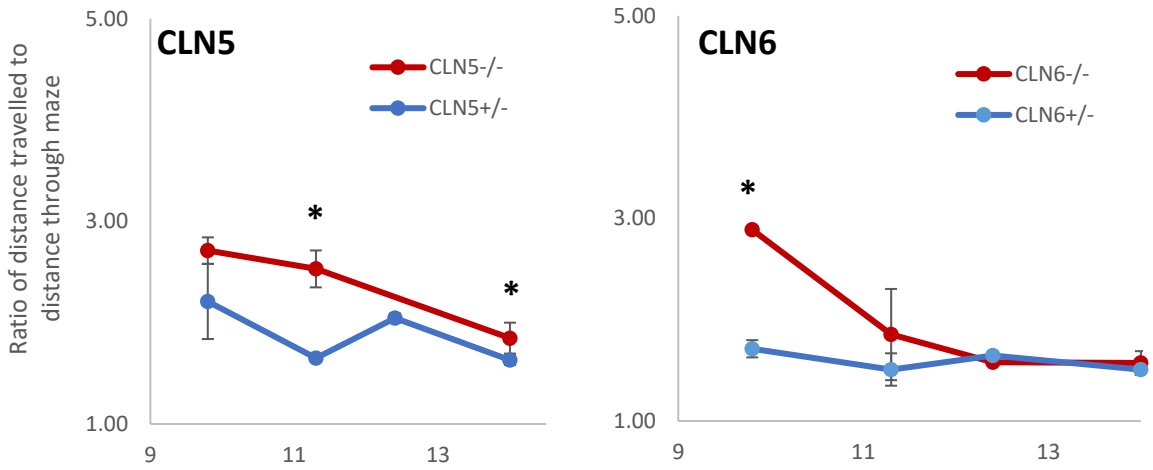

Figure 3-10. Mean (+/- sem) ratios of distance travelled to linear distance through the reverse maze for CLN5 and CLN6 normal and affected cohorts. Asterisks denote significant differences (P<0.05).

The CLN6$^{-/-}$ affected cohort took a significantly (P<0.05) longer path on their first time through the reverse maze, at 9.8 months, than the CLN6$^{-/-}$ controls. However there was no significant (P>0.05) difference between the affected and normal CLN6 cohorts when tested at the later ages (11.3, 12.4 or 14 months).

	Age (months)			
	9.8	11.3	12.4	14
Tag #		*CLN5$^{-/-}$ affected*		
1119	nd	2.25	nd	1.81
1125	2.84	2.76	nd	1.65
1142	2.58	2.57	nd	2.08
		CLN6$^{-/-}$ affected		
1027	nd	2.57	unable	
1046	nd	1.37	1.58	1.46
1047	2.89	1.61	nd	1.69

Table 3-7. Mean ratios of distance travelled to linear distance through the reverse maze for individual CLN5$^{-/-}$ and CLN6$^{-/-}$ affected animals. Red figures indicate significantly (95% c.i.) slower than normal control cohort. nd = no data, due to failure of GPS unit.

The individual data for the CLN5$^{-/-}$ affected control cohort (Table 3-7) showed that all three animals took a significantly ($P<0.05$) longer route than the normal controls at the 11.3 month testing. This was mostly attributable to normal control animals taking a shorter path at that time (Figure 3-10), and overall CLN5 affected animals got slightly better at following a more direct route over time. Data were only available for one of the CLN6 affected cohort (1047), at the first time point (9.8 months), and it took a significantly (95% c.i.) longer route through the maze than normal controls. The CLN6 affected animal, 1027, also took a significantly (95% c.i.) longer route at 11.3 months, and by 12.4 months was unable to traverse the maze.

3.3.10 Reverse maze transit times of the CLN5$^{-/-}$ treated cohort

Maze traverse times for the three CLN5$^{-/-}$ cohorts that received sc AAV9 CLN5 treatment at 3, 6 or 9 months of age were plotted against the normal and untreated CLN5$^{-/-}$ sheep data. Results are presented in Figure 3-11. The six animals treated at 3 months recorded faster traverse times than the mean affected control cohort until final testing at 14 months of age. When 3 month treated individuals were compared to the normal cohort (Table 3-8) few were significantly slower over the duration of the experiment. Only treated animals 1104 and 1111 were significantly (95% c.i.) slower at 11.3 months and 1120 at 14 months. The 6 month treated cohort did not respond as

well to the reverse maze task as those treated at 3 months, with two of the three (1185 and 1186) being significantly (95% c.i.) slower at 14 months of age.

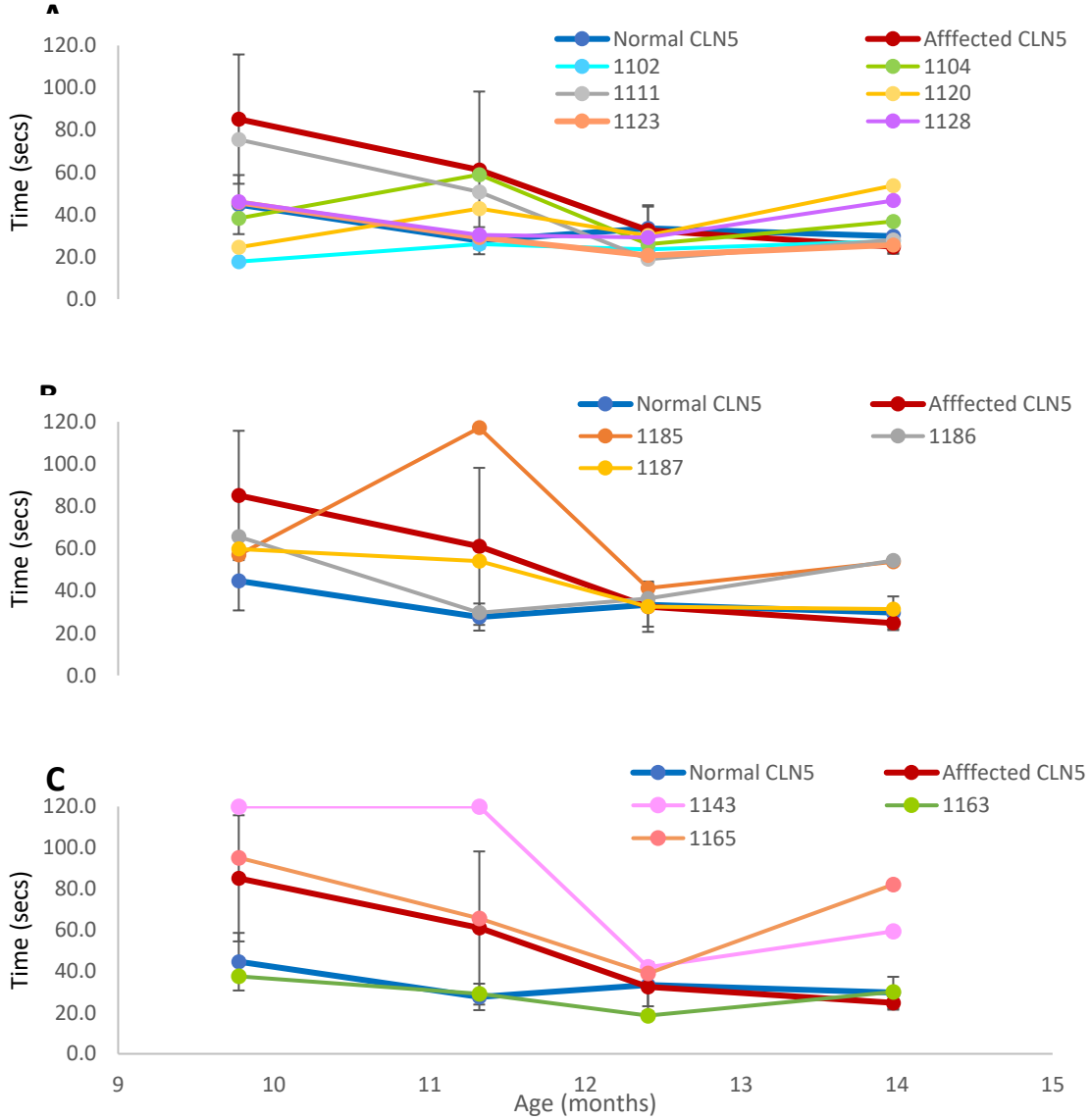

Figure 3-11. Reverse maze traverse times for individual CLN5$^{-/-}$ sheep treated by gene therapy at 3 months (A), 6 months (B) or 9 months (C), plotted against mean times for normal (blue line) and affected (red line) controls.

One of the 9 month treated cohort (1143) failed to traverse the reverse maze at 9.8 and 11.3 months of age, and was significantly (95% c.i.) slower than the mean of the normal controls at 14 months. Of the other two animals in the 9 month treated cohort, animal 1165 was significantly (95% c.i.) slower than the mean of the normal control cohort at both 11.3 and 14 months of age, whilst 1163 was as fast as a normal sheep at all times.

	Age (months)			
	9.8	11.3	12.4	14.0
CLN5$^{-/-}$ treated at 3 months				
1102	17.7	26.0	23.5	27.5
1104	38.2	58.9	26.0	36.7
1111	75.5	50.6	18.9	28.0
1120	24.5	42.9	30.0	53.7
1123	45.8	29.1	20.7	25.7
1128	46.2	30.4	29.2	46.6
CLN5$^{-/-}$ treated at 6 months				
1185	57.1	117.1	41.3	53.8
1186	65.7	29.6	36.5	54.4
1187	59.8	54.1	32.6	31.4
CLN5$^{-/-}$ treated at 9 months				
1143	120	120	42.0	59.5
1163	37.6	29.2	18.6	30.1
1165	95.2	65.7	39.0	82.2

Table 3-8. Reverse maze mean traverse times for individual CLN5$^{-/-}$ affected and CLN5$^{-/-}$ animals treated by gene therapy at 3, 6 or 9 months of age. Red figures indicate significantly slower traverses (95% c.i.) than normal control cohort.

3.3.11 Reverse maze distance ratios for CLN5$^{-/-}$ treated cohorts

The ratio of distance travelled to linear distance for the CLN5$^{-/-}$ animals who received brain-directed sc AAV9 CLN5 treatment at 3, 6 or 9 months was plotted against the mean normal and untreated CLN5$^{-/-}$ sheep data Figure 3-12. As with the time data, the majority of the distance ratios for the animals treated at 3 and 6 months were shorter than for the affected control cohort. Again the 9 month treated data were more variable. Analysis of individual data (Table 3-9), showed that all animals in the untreated affected and 9 month treated cohorts followed a significantly longer path at 11.3 months (than normal controls). This was likely due to better performance of normal controls at that time rather than deterioration of performance of the affected and treated animals.

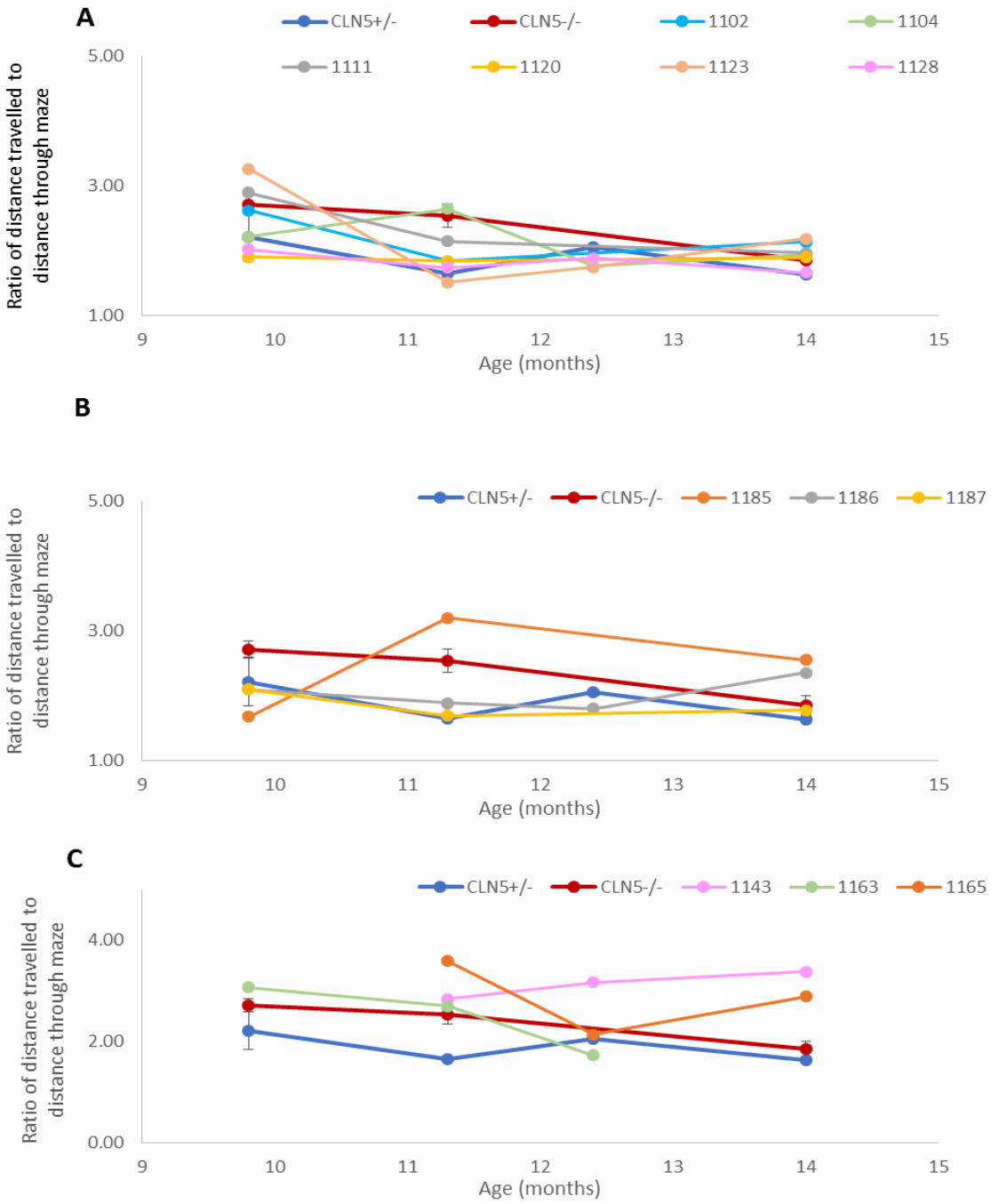

Figure 3-12. Ratios of distance travelled to linear distance through reverse maze for individual CLN5$^{-/-}$animals treated by gene therapy at 3 (A), 6 (B) or 9 (C) months of age, plotted against mean normal controls (blue line) and mean affected controls (red line).

	Age (months)			
	9.8	11.3	12.4	14
	CLN5$^{-/-}$ affected			
1119	nd	2.25	nd	1.81
1125	2.84	2.76	nd	1.65
1142	2.58	2.57	nd	2.08
	CLN5$^{-/-}$ treated @ 3 mth			
1102	2.62	1.83	nd	2.13
1104	2.21	2.64	1.75	1.93
1111	2.89	2.14	nd	1.96
1120	1.89	1.83	nd	1.89
1123	3.26	1.51	1.74	2.18
1128	2.01	1.73	1.88	1.66
	CLN5$^{-/-}$ treated @ 6 mth			
1185	1.67	3.20	nd	2.55
1186	2.09	1.89	1.79	2.35
1187	2.09	1.69	nd	1.77
	CLN5$^{-/-}$ treated @ 9 mth			
1143	nd	2.84	3.17	3.38
1163	3.07	2.70	1.73	nd
1165	nd	3.59	2.14	2.89

Table 3-9. Ratios of distance travelled to linear distance through reverse maze for CLN5$^{-/-}$ affected and CLN5$^{-/-}$ animals treated at 3, 6 or 9 months of age. Red figures indicate significantly (P<0.05) longer paths then normal controls. nd = no data (due to GPS unit failure).

3.3.12 Reverse maze traverse times of the CLN6$^{-/-}$ treated cohort

Reverse maze traverse times for the CLN6$^{-/-}$ cohort that received gene therapy at 3 months of age were plotted against the normal and untreated CLN6$^{-/-}$ sheep data. Results are presented in Figure 3-13 and Table 3-10. The treated cohort were slow for the initial maze runs at 9.8 months, with 5 of the six animals significantly (95% c.i.) slower than normal controls. After this there was only one treated animal (1014) that was significantly (95% c.i.) slower than the mean normal time at 14 months of age.

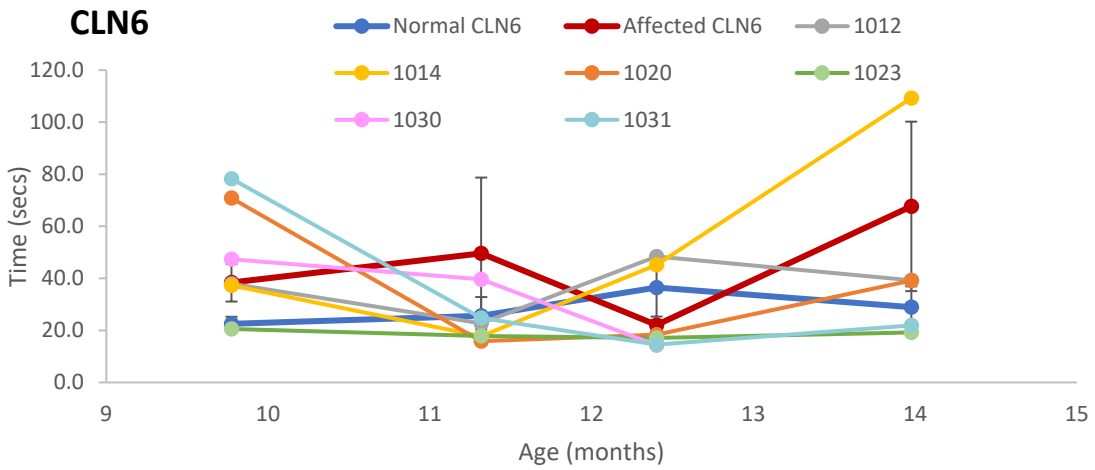

Figure 3-13. Reverse maze traverse times for individual CLN6$^{-/-}$ sheep treated by gene therapy at 3 months, plotted against mean times for normal (blue line) and affected (red line) controls.

	Age (months)			
	9.8	11.3	12.4	14.0
CLN6$^{-/-}$ affected				
1027	47.3	96.7	nd	120
1046	27.1	20.0	20.2	33.5
1047	40.7	32.0	24.0	49.5
CLN6$^{-/-}$ treated at 3 months				
1012	38.0	22.7	48.4	39.3
1014	37.4	17.8	45.1	109.3
1020	70.8	15.9	18.4	39.2
1023	20.6	17.9	17.1	19.3
1030	47.3	39.7	14.3	
1031	78.3	24.7	14.6	21.9

Table 3-10. Reverse maze mean traverse times for individual CLN6$^{-/-}$ affected and CLN6$^{-/-}$ animals treated by gene therapy at 3 months of age. Red figures indicate significantly slower traverses (95% c.i.) than those of the normal cohort.

3.3.13 Reverse maze distance ratios for CLN6$^{-/-}$ treated cohorts

The ratio of distance travelled to linear distance for the CLN6$^{-/-}$ animals who received brain-directed gene therapy at three months were plotted against the untreated CLN6$^{-/-}$ affected and CLN6$^{+/-}$ normal controls (Figure 3-14). The individual data Table 3-11 showed the untreated control (1047) and four of the six treated animals were significantly (95% c.i.) slower than the normal sheep on their first attempt at the reverse maze. When last tested at 14 months, one of the untreated cohort (1027) could no longer transit the maze, and another (1047) took

significantly (95% c.i.) longer route than the normal cohort. Similarly the same four out of five of the treated cohort were taking a significantly (95% c.i.) longer route at this time. Only treated animal 1023 remained on a similar path length to normal animals.

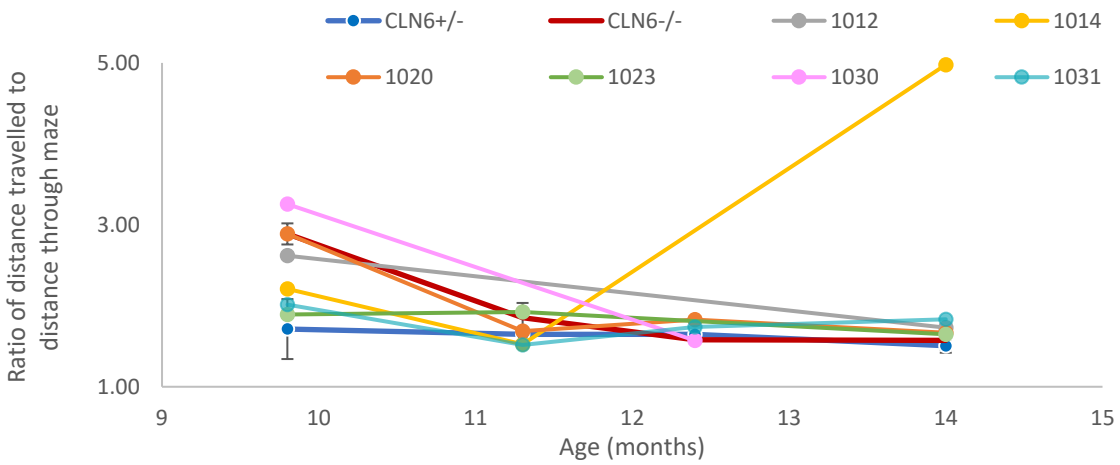

Figure 3-14. Ratio of distance travelled to linear distance through reverse maze for individual CLN6$^{-/-}$ sheep treated by gene therapy at 3 months, plotted against mean times for normal (blue line) and affected (red line) controls.

	Age (months)			
	9.8	11.3	12.4	14
CLN6$^{-/-}$ affected control				
1027	no data	2.57	unable	unable
1046	no data	1.37	1.58	1.46
1047	2.89	1.61	no data	1.69
CLN6$^{-/-}$ treated at 3 mth				
1012	2.62	no data	no data	1.73
1014	2.21	1.52	no data	4.98
1020	2.89	1.69	1.83	1.67
1023	1.89	1.92	no data	1.65
1030	3.26	no data	1.57	no data
1031	2.01	1.52	1.74	1.83

Table 3-11. Ratios of distances travelled to linear distance through reverse maze for individual CLN6$^{-/-}$ sheep and CLN6$^{-/-}$ sheep treated by gene therapy at 3 months. Red figures indicate significantly differences (95% c.i.) to the normal cohort.

3.3.14 Data summary for CLN5$^{-/-}$ untreated and treated cohorts

The ERG, ICV and clinical outcome for the CLN5$^{-/-}$ untreated and treated animals is summarised in Table 3-12.

Sheep	Genotype	Treatment	Minimal ERG (months)	ICV changes over time	Clinical outcome
1119	CLN5$^{-/-}$	None	n.t	n.t	Euthanised at 16.4 m, blind, advanced disease
1125	CLN5$^{-/-}$	None	n.t	n.t	Euthanised at 16.3 m, blind, advanced disease
1142	CLN5$^{-/-}$	None	n.t	n.t	Euthanised at 16.2 m, blind, advanced disease
1102	CLN5$^{-/-}$	scAAV9.CLN5 at 3 m (LD)	25	+12.5 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1104	CLN5$^{-/-}$	scAAV9.CLN5 at 3 m (HD)	19.8	-2.5 mL (3 – 27 m)	Euthanised at 27.4 m, blind, moderate clinical disease
1111	CLN5$^{-/-}$	scAAV9.CLN5 at 3 m (LD)	18.8	+3.1 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1120	CLN5$^{-/-}$	scAAV9.CLN5 at 3 m (HD)	18.8	+9.6 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1123	CLN5$^{-/-}$	scAAV9.CLN5 at 3 m (HD)	18.8	+5.0 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1128	CLN5$^{-/-}$	scAAV9.CLN5 at 3 m (LD)	18.7	+9.6 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1185	CLN5$^{-/-}$	scAAV9.CLN5 at 6 m (HD)	18.1	-5.3 mL (3 – 21 m)	Euthanised at 21.3 m, blind, moderate clinical disease
1186	CLN5$^{-/-}$	scAAV9.CLN5 at 6 m (HD)	18.1	-1.1 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1187	CLN5$^{-/-}$	scAAV9.CLN5 at 6 m (HD)	18.1	-0.4 mL (3 – 31 m)	Alive at 31 m, blind, no other disease symptoms
1143	CLN5$^{-/-}$	scAAV9.CLN5 at 9 m (HD)	18.6	-6.4 mL (3 – 18 m)	Euthanised at 18.5 m, blind, advanced disease
1163	CLN5$^{-/-}$	scAAV9.CLN5 at 9 m (HD)	18.4	+4.4 mL (3 – 31 m)	Euthanised at 30.7 m, blind, moderate clinical disease
1165	CLN5$^{-/-}$	scAAV9.CLN5 at 9 m (HD)	19.5	-7.4 mL (3 – 21 m)	Euthanised at 21.6 m, blind, moderate clinical disease

Table 3-12. Data summary of ERG, ICV and clinical data collected on individual CLN5 animals. Abbreviations: n.t, not tested; LD, low dose 1.65x10^{12} vg; HD, high dose 5x10^{12} vg; vg, viral genomes; m, months.

The untreated affected cohort were all euthanized at just over 16 months with advanced disease. The treated cohorts had better outcomes, evident with all of the efficacy measures, and better the earlier they were treated which allowed extension of times to euthanasia. Five of the six animals that were treated at three months, and 2/3 of the animals treated at 6 months were still alive at 31 months, and apart from blindness were showing no other symptoms. The cohort treated at 9 months had the poorer outcomes, although one animal survived to 21.6 months and another to 30.7 months, considerably longer than the untreated cohort. Treatment did not improve ERG loss, all other animals losing ERG response at 18-19 months, although one of the 3-month treated cohort (1102) retained an ERG response until 25 months. The ERG, ICV and clinical outcome for the CLN6$^{-/-}$ untreated and treated animals is summarised in Table 3-13.

3.3.15 Data summary for CLN6$^{-/-}$ untreated and treated cohorts

Sheep	Genotype	Treatment	Minimal ERG (months)	ICV changes over time	Clinical outcome
1027	CLN6$^{-/-}$	none	n.t.	n.t.	Euthanised at 16.4 m, blind, advanced disease
1046	CLN6$^{-/-}$	none	n.t.	n.t.	Euthanised at 16.1 m, blind, advanced disease
1047	CLN6$^{-/-}$	none	n.t.	n.t.	Euthanised at 16.1 m, blind, advanced disease
1012	CLN6$^{-/-}$	scAAV9.CLN5 at 3 m (LD)	16.5	-0.5 mL (3 – 17 m)	Euthanised at 19.5 m, advanced disease
1014	CLN6$^{-/-}$	scAAV9.CLN5/6 at 3 m (HD)	11.2	-4.8 mL (3 – 17 m)	Euthanised at 19.0 m, advanced disease
1020	CLN6$^{-/-}$	scAAV9.CLN5 at 3 m (LD)	18.1	-10.0 mL (3 – 17 m)	Euthanised at 19.5 m, advanced disease
1023	CLN6$^{-/-}$	scAAV9.CLN5/6 at 3 m (HD)	19.0	-7.1 mL (3 – 17 m)	Euthanised at 18.9 m, advanced disease
1030	CLN6$^{-/-}$	scAAV9.CLN5 at 3 m (HD)	17.9	-3.6 mL (3 – 17 m)	Euthanised at 22.1 m, advanced disease
1031	CLN6$^{-/-}$	scAAV9.CLN5/6 at 3 m (LD)	18.8	-6.8 mL (3 – 17 m)	Euthanised at 22.1 m, advanced disease

Table 3-13. Summary of the ERG, ICV and clinical data collected on individual CLN6 animals. Abbreviations: n.t, not tested; LD, low dose 1.65x1012 vg; HD, high dose 5x1012 vg; vg, viral genomes

The untreated affected cohort were all euthanized at 16.1 – 16.4 months with advanced disease. Treatment did not prevent the disease, with all treated animals ultimately succumbing, but likely delayed its onset, progression, and extended the lifespan of the animals in this study by 2-4 months. There was minimal ICV loss in one animal (1012) compared to a previous study (K. Russell, 2017) which found an average of 5.8 ml loss between 3- 17m in untreated CLN6$^{-/-}$ animals.

3.4 Discussion

This study established that sheep were able to navigate the more complex maze that had been developed in Experiment 1. Three key trends were apparent. Firstly, the CLN5 Borderdales were often slightly slower than the CLN6 South Hampshires, but when using the ratio of the distance travelled through the maze to the linear distance, the performance of the normal cohort of both breeds was the same. Secondly, all animals were slowest and took their longest paths when first exposed to the maze, but were faster and took shorter paths in subsequent transits. This would appear to show the learning, and memory described in many other studies with sheep in mazes (C.V. Bazely D.R. Ensor, 1989; Camm et al., 2000; Hunter et al., 2015; Johnson et al., 2012; McBride et al., 2016; Morton & Avanzo, 2011). Finally, the untreated affected cohorts of both genotypes were generally slower and took a longer path length through the maze than their unaffected counterparts. Due to the variability between animals, and the small numbers (n=3) in the cohorts, this often did not reach significance. However the loss of ability of individual affected (both untreated and treated) animals to negotiate the maze correlated well with other measures of disease progression, from other concurrent studies on the same animals (Tables 3-12 and 3-13).

3.4.1 CLN5 genotype

The Borderdale sheep is a breed that appears to habituate to human contact quite readily (Erhard et al., 2006). In this study, where escape from the operator is one of the drivers to negotiate the maze, the readiness of Borderdale sheep to stop when about 10 metres from the operator was very apparent, and has been noted by other researchers working with this breed (McBride et al., 2016). This means that the time taken to negotiate the maze is not a good measure of ability for these animals, as the data is very variable, and in this study although the untreated CLN5 affected controls appeared to be generally slower than the normal CLN5 cohort through both the normal and reversed maze, they were rarely significantly slower. The path length is a more informative measure evident in the fact that the CLN5 affected cohort always took a longer path than the normal controls, reaching significance ($P<0.05$) at five of the eight time points, and significance at $P<0.1$ at the other times. When the maze was reversed the paths of the affected cohort were also significantly ($P<0.05$) longer at the final two times of 11.3 and 14 months.

The time taken for the treated CLN5 Borderdale cohorts to transit the maze were quite variable. However conclusions could still be drawn on the validity of maze testing in the assessment of efficacy for the gene therapy trials. The $CLN5^{-/-}$ treated at 3 months of age responded well to treatment and performed well in both forms of the maze, and by both measures. There are occasions where individual animals would be significantly slower, or take a significantly longer path than the normal controls, but these were seldom, and randomly scattered over the experiment. There was no indication that the animals were deteriorating over the course of the study. The efficacy of the treatment was also reflected in the other measures of disease progression, such as ICV and the oBDRS. The majority (5/6) of this cohort were still alive at 31 months, as opposed to the untreated $CLN5^{-/-}$ cohort who were euthanised at 16 months with advanced disease. The animals treated at 6 months also responded well to treatment, with two still alive at 31 months with no symptoms other than blindness. The animal (1185) that lost most

ICV and was euthanised at 21 months with moderate disease, performed as well as the other two in the cohort in the normal maze, but not as well in the reversed maze. Later treatment was not as effective and the performance of the cohort treated at 9 months was mixed. Animal 1143 lost ICV and was euthanised with advanced disease at 18.5 months. This animal was the poorest performer in the 9 month treated cohort in both configurations of the maze. The maze performance of the other two animals in the cohort was inconsistent. Animal 1163 performed well when time was used as a measure in both the normal and reversed mazes, and clinically by the ICV and longevity, but the distance ratios for this animal were consistently longer than those of the normal controls. The final animal 1165 performed better than 1143 but not as well as 1163, and this was reflected in the clinical data for the animal. Overall the maze data were a good reflection of the variable response to treatment by this group.

3.4.2 CLN6 genotype

As was found in Experiment 1, time was a good measure of CLN6 South Hampshire's ability in the maze. The South Hampshire breed does not appear to acclimate to the human contact as quickly as the Borderdales, and retained the desire to get away from the operator, and be reunited with their conspecifics. The affected CLN6 cohort was significantly ($P<0.05$) slower than controls for all but one of the times between 5.2 and 14 months. The one time (12.4 months), where the mean of the affected cohort was not slower than the normal controls (Figure 3-7), was when the affected animal 1027 which normally struggled to transit the maze is missing from the data. Hence the CLN6$^{-/-}$ affected cohort was skewed by this animal. It was consistently significantly ($P<0.05$) slower than the normal controls throughout the whole of the study, and also much slower than the rest of the CLN6$^{-/-}$ affected cohort. The distance ratio showed the CLN6$^{-/-}$ affected cohort took a significantly ($P<0.05$) longer route at first exposure to the maze (5.2 months), and then again at 7.4 months, but ratios for the rest of the study were very similar for both the normal and reversed maze.

It can be seen from the clinical data that the CLN6 treatment was not as successful as treatment for the CLN5. This was not reflected in the times taken to transit the maze, although the treated cohort did perform particularly poorly when exposed to the reverse maze for the first time. Five of the six in the treatment group took a significantly longer time to negotiate the maze at the first exposure. The results for distance travelled show that 4 out of 5 animals were taking a significantly longer path for both the normal and the reversed maze when last tested at 14 months.

Overall this experiment did not achieve the objective of being able to discern the onset of Batten disease at an early stage. The fact that all the affected animals of both genotypes performed less well than normal animals when first exposed to the maze in either configuration formed the basis for the next experiment. It was decided to use a maze that was more biased to the executive function of sheep, rather than spatial learning.

4 Experiment 3

4.1 Introduction

The two-choice Y or T type maze for testing spatial learning and ability to associate visual cues with correct paths, or food rewards, has been widely used with sheep. Mazes without visual cues have been used to test the sheep's ability to learn a path through a maze, generally with a food reward. Some of these studies have been proof of concept studies to test learning ability (Hunter et al., 2015; Johnson et al., 2012). The effects of prenatal undernutrition (Erhard et al., 2004; Hernandez et al., 2009), litter size and sex (Hernandez et al., 2009), age (Johnson et al., 2012) and experience (Hunter et al., 2015) have all been tested on the ability of sheep to learn a path. It has been known for some time that sheep are able to recognise a variety of visual cues. The ability to associate the visual cue of different commonly encountered plant types with a preferred type of feed pellet has been shown (Edwards et al., 1997). Sheep are able to discriminate colour brightness and hue (C.V. Bazely D.R. Ensor, 1989) and recognise other individual sheep of their own breed (Kendrick et al., 1995), although this ability was very variable between sheep breeds. Sheep have also been trained to associate pictures of a particular 3 month old lamb with a food reward, and then transfer that knowledge to pictures of the same lamb at one month of age (Ferreira et al., 2004). Furthermore this has been shown to be remembered in many animals up to 2 years later (Keith et al., 2001). The effects of prenatal undernutrition have also been studied with a T –maze (Camm et al., 2000) with the maternal ewe as the visual cue. The ability to visually discriminate between symbols in a maze has also been demonstrated (McBride et al., 2016; Morton & Avanzo, 2011). Once trained to associate a symbol or colour with a food reward, sheep were also able to learn a reversal of this association (Johnson et al., 2012; McBride et al., 2016; Morton & Avanzo, 2011).

In light of these studies it was decided to trial an adaptation of symbol recognition and reversal learning with the Batten disease affected animals in an attempt to be able to discriminate between the diseased and healthy unaffected states at an earlier time than was achieved with the field maze.

4.2 Materials and methods

The learning maze was set up in the same area as the previous maze, and occupied the same dimensions. It had three gates, and at each one the animal was presented with a choice of two alternative routes around the gate (*Figure 3-15*). Only one side was open to allow the animal passage through the maze, and this side was always associated with a green coloured feed bin and a large plus (+) symbol on a piece of (700 x 900mm) sized corflute plastic (*Figure 3-16*). The other side of the gate was blocked off and was always associated with a yellow feed bin and a triangular (Δ) symbol on a large plastic sign.

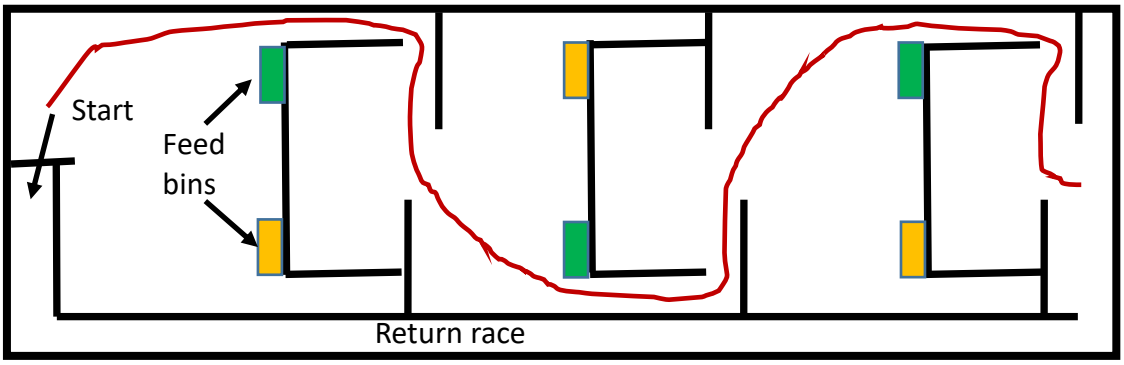

Figure 3-15. Layout of learning maze.

The animals had been habituated to the green bin and plus sign being associated with food, by regularly feeding of lucerne pellets (Farmlands Nutrition, P O Box 31, Rolleston New Zealand) to them in green bins associated with + signs in the field. The yellow bins and triangular signs were always associated with no food. The animals were followed through the maze by the operator, and were gauged to have completed a particular configuration of the maze when they made

three consecutive traverses without an error. Errors occurred when the animal entered the blocked off race indicated by the yellow feed bin. If the animal entered the error race, a plastic bag on a stick was waved at the animal as it exited. The animals were scored on the number of runs required before it had completed 3 consecutive error free runs, and the maximum score was 24 runs. If the animal had completed 24 runs with a particular configuration without 3 consecutive error free runs, then it was moved on to the next configuration.

Figure 3-16. Photo of two choice learning maze, showing green bins and + signs associated with the correct course through the maze.

When an animal was tested, it was run through the maze in four different configurations, always in the same order. The maze was initially run as a zig-zag with the "correct" path starting to right of first gate (*Figure 3-15*). When the animal had completed that set-up the maze was reversed to be a zig-zag with the correct path starting to the right. Then it was changed so that the correct path was to the right of all gates, then reversed so it was to the left of all gates.

4.2.1 Sheep

Four groups of three animals were tested. The groups were CLN5$^{+/-}$ normal (n=3), CLN5$^{-/-}$ affected (n=3), CLN6$^{+/-}$ normal (n=3) and CLN6$^{-/-}$ affected (n=3). The animals were tested twice at an average age of 5.3 (min 5.1, max 5.7) and 6.4 months (min 6.2, max 6.8).

4.2.2 Statistical techniques

Means and corresponding SEM was calculated for each group at each time point. Student's t-tests were performed to test each group against the normal controls at each time point. Differences were regarded as significant where $P<0.05$. Where individual treated and untreated affected animals were compared to the control group, they were considered to be significantly different from controls when they were more than 1.96 standard deviations away from normal control mean values.

4.3 Results

The mean number of runs required by each cohort to complete all four variants of the maze was calculated and the values for the normal controls (heterozygotes; $^{+/-}$) plotted against the affected controls (homozygous recessive; $^{-/-}$). The CLN5$^{-/-}$ and CLN6$^{-/-}$ affected controls took significantly ($P<0.05$) more runs to complete all four maze variants at 6.4 months of age, but not at 5.3 months (*Figure 3-17*).

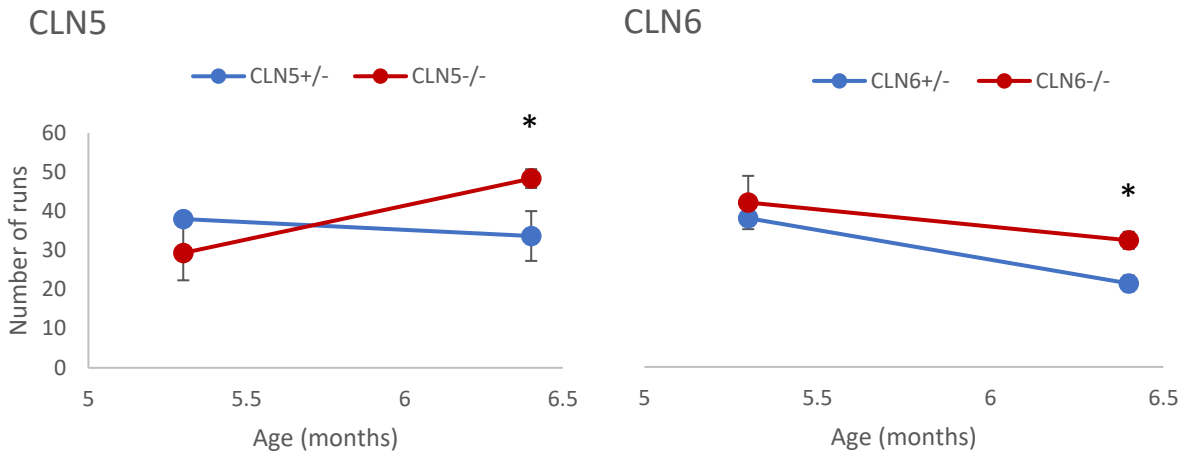

Figure 3-17. Mean (+/- sem) number of runs for both CLN5 and CLN6 cohorts required to complete all four variants of the maze. Asterisk denotes significantly (P<0.05) different values.

The number of runs required for each individual to complete each variant of the maze was plotted. There was no significant difference (p<0.05) between the means of the normal and affected cohorts for either genotype for any configuration of the maze at any time, and individual data were highly variable (*Figure 3-18*).

	Age				Age
CLN5+/-	5.3	6.4	CLN6+/-	5.3	6.4
1104	37	39	1003	34	18
1116	41	41	1005	40	25
1164	36	21	1009	40	21
mean	**38**	**33.7**	**mean**	**38**	**21.3**
CLN5-/-			CLN6-/-		
1146	20	53	1045	39	34
1147	25	45	1048	55	35
1149	43	47	1049	32	28
mean	**29.3**	**48.3**	**mean**	**42**	**32.3**

Table 3-14 Total number of runs required to negotiate all 4 configurations of the maze for individual sheep. Red figures denote totals for affected sheep that are significantly (95% c.i.)

slower than the normal control cohort.

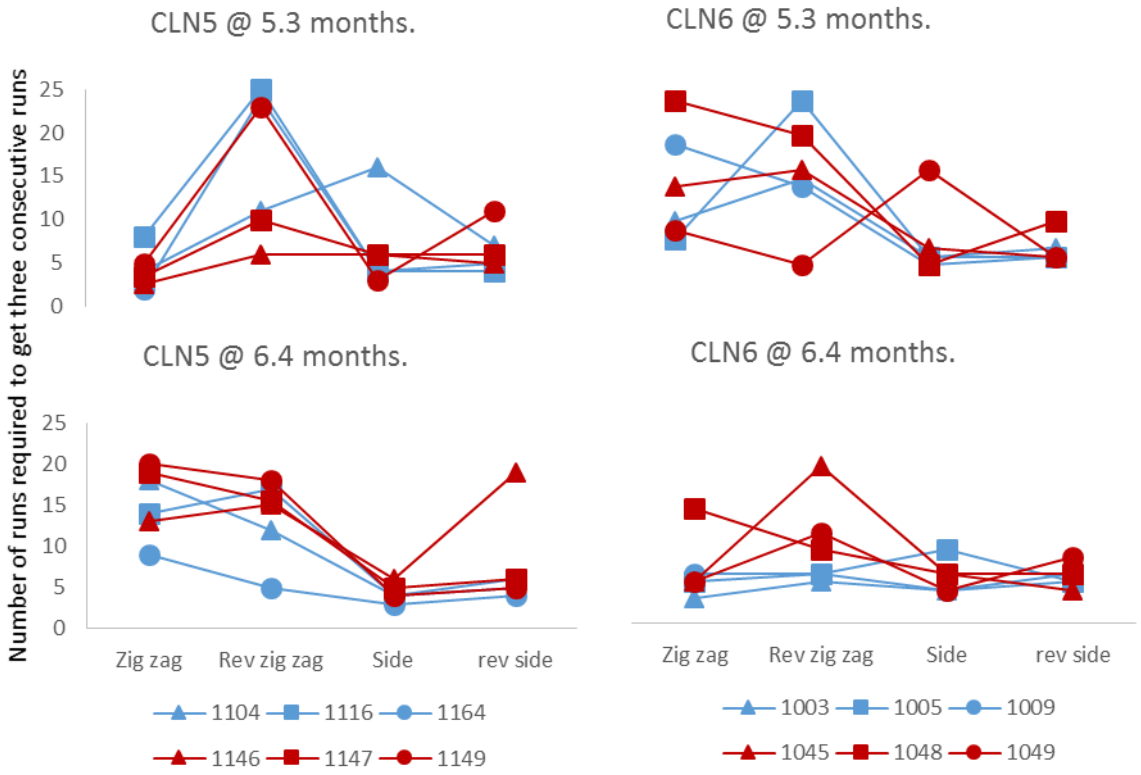

Figure 3-18. Number of runs required to get three consecutive error free runs, for individual normal (blue lines) and affected (red lines) animals of both genotypes at 5.3 and 6.4 of age.

The affected individuals of both genotypes were compared with the normal control cohorts (*Table 3-15*) and the number of times the individuals required significantly more attempts recorded. The CLN5$^{-/-}$ affected cohort only required significantly more runs on one occasion when 5.3 months and three occasions when 6.4 months of age. The CLN6$^{-/-}$ affected cohort took more runs on four occasions at 5.3 months, and three at 6.4 months. There was no obvious pattern on which configuration the affected animals found more difficult than the normal cohort.

5.3 months of age	6.4 months of age

	maze configuration							
	Zig zag	Rev zig zag	Side	rev side	Zig zag	Rev zig zag	Side	rev side
				CLN5$^{-/-}$ affected				
1146	3	6	6	5	13	15	6	19
1147	3	10	6	6	19	15	5	6
1149	5	24	3	11	19	18	4	6
				CLN6$^{-/-}$ affected				
1045	13	15	6	5	5	19	6	4
1048	23	19	4	9	14	9	6	6
1049	8	4	15	5	5	11	4	8

Table 3-15. The number of runs required for individual affected animals to complete 3 error free runs, for each configuration of the maze. Red figures indicate that affected animals required significantly (P<0.05) more runs than normal controls.

4.4 Discussion

This two-choice maze test was only run at two time points (5.3 and 6.4 months) at an early stage in the progression of the disease. These earlier time points were used as the aim of this more cognitively complex maze, was to discriminate between a normal or affected animal earlier than the two previous mazes. The two-choice maze demonstrated that at 6.4 months of age the affected cohorts of both genotypes took significantly more runs to complete all conformations of the maze than the normal control cohorts.

All affected individuals of both genotypes also took more runs to complete all conformation than the normal controls at 6.4 months, but due to the variability of the normal control groups, the extra runs required by the individual CLN5$^{-/-}$ did not reach significance, and only reached significance with two of the three CLN6$^{-/-}$. However, there was no pattern in the different individual conformations that the affected animals found more difficult to negotiate, and none of the affected animals consistently took significantly more runs through the various maze conformations.

The animals used in this experiment were quite young, and this may have had a bearing on their ability in the maze. Previous published two-choice maze studies have been on sheep of various

ages, including 4 months (Hernandez et al., 2009), 6 months (Doyle et al., 2014), 11-18 months (Taylor et al., 2010), 12 months (Morton & Avanzo, 2011), eighteen months (Erhard et al., 2004; Hernandez et al., 2009), 24 months (Lee et al., 2006), 24-36 months (McBride et al., 2016) and over 36 months of age (Ferreira et al., 2004; Peirce et al., 2001). Previously researchers have found that 3.5 month old lambs performed markedly better than 2.5 month old lambs in a non-matching-to–place, position habit and position habit reversal tasks, and remarked that the age at which performance is optimised has to yet be determined (Johnson et al., 2012). However (Hunter et al., 2015) found no significant difference between naive animals of 4.5 or 10 months of age for the majority of their maze learning and reversal tasks, and in fact 10 month old naive females were slower to work out the first reversal of the maze.

The cumulative stress on the animals in this experiment, caused by naivety (this being their first exposure to a maze), getting stuck or the negative reinforcement of the waved plastic bag may also have affected the animals' abilities. A study by (Doyle et al., 2014) found that exposure to a dog, accompanied by barking, caused sheep to make marginally significantly (P=0.057) more errors when traversing the maze on the first occasion, but not when subsequently exposed to the same stressor. Another study on the effects of prenatal undernutrition, found that lambs born to ewes that had undergone restricted nutrition, were more emotionally reactive and had stronger reactions to adverse events(Erhard et al., 2004). These animals were also slower to learn reversals of the maze. Whilst the effects were not dramatic in either of these studies, the potential of stress to influence the sheep's ability should be taken into account when designing a testing protocol.

Many of the previous studies involving sheep in mazes have included habituation and training periods prior to the experiment. As an example, the study by (Johnson et al., 2012) on T-maze learning in 3.5 month old lambs, involved an acclimatization of 15 exposures to the maze where the animals were fed inside the maze. This was followed by a training period of 45 runs through

the maze to food rewards. These animals had also been housed inside, and fed twice a day by the handlers. The current trial probably most resembles that carried one that also involved sheep following a path through a two-choice maze with the correct path indicated by symbols or colours (Morton & Avanzo, 2011). In that trial the sheep were habituated to human handling over a period of 4 months, and then to the maze by five 5-10 minute exposures on five separate occasions. The 7 animals then required 56 discriminations to reach a criterion of 80% correct in 16 discriminations (12 correct choices in 16 attempts). When the colour was reversed the number of discriminations required to reach the criterion increased to 88, and the time required to put 8 sheep through those two mazes was 9 days, as time to negotiate was not a parameter. The study by Morton and Avanzo (2011) was a 'proof of concept' and hence used normal sheep, and required a huge investment of time. Studies involving animals that have any form of hindrance to their ability (e.g. Batten disease affected sheep) could be expected to take longer to complete the studies. The current study included a period of acclimatisation to the green bin containing food within the field, and being associated with the + symbol, but this was not geographically in the context of the maze.

5 Final conclusions

The mazes used in this study were able to discern the onset and progression of Batten disease, and maze failure correlated with other *in vivo* measures of disease progression already in use. The difference in temperament between the two breeds used in this study meant that recording both the time taken, and the distance covered compensated for these differences. Time being a good measure for the South Hampshire breed, with the more 'flighty' disposition, whereas the distance covered was a better measure for the Borderdale, with its more placid and equable nature. Successful gene therapy treatment was reflected in improved maze performance, but as the treatment did not include the eyes, the animals ultimately failed the maze due to loss of eyesight. However none of the maze tests utilised were able to detect disease onset any earlier than the methods currently in use. Despite not being able to assist in early disease diagnoses, maze testing was a useful adjunct method to assess efficacy in sheep who had received CLN5 or CLN6 gene therapy.

Much of the published work with sheep and mazes have been proof-of-concept studies using normal animals. In many of these studies there has been a selection of animals, so animals unable to complete the task have been excluded. As an example, in a similar two-choice maze using visual discrimination of symbols only 64% (9/15 animals) of the Borderdale sheep completed the study (McBride et al., 2016). Another study looking at recognition of faces and symbols found that many animals were unable to make the discrimination, and also found large breed differences, with one breed much more capable than the other(Kendrick et al., 1995). This infers that there is a large amount of variability in the ability of sheep to learn and negotiate mazes, which has been reinforced in this study. This individual variability when using cohorts of three animals, as in the current study, mean that statistical significance is hard to achieve. There also appears to be a large amount of variability within animals over time, which also makes interpretation of results difficult.

Many of the cognitively more difficult tasks that researchers have used require large amounts of time devoted to habituation, training and selection of animals capable of the task. The amount of time required was beyond the scope of this study, and selection of animals post treatment but these more cognitively demanding tasks may still have potential as a method of early diagnosis in ovine Batten disease, if the man hours required are available.

6 References

Anderson, G. W., Goebel, H. H., & Simonati, A. (2013). Human pathology in NCL. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1832*(11), 1807–1826. https://doi.org/10.1016/j.bbadis.2012.11.014

Bond, M., Holthaus, S.-M. kleine, Tammen, I., Tear, G., & Russell, C. (2013). Use of model organisms for the study of neuronal ceroid lipofuscinosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1832*(11), 1842–1865. https://doi.org/10.1016/J.BBADIS.2013.01.009

C.V. Bazely D.R. Ensor. (1989). Discrimination learning in sheep with cues varying in brightness and hue. *Applied Animal Behaviour Science*, *23*, 293–299.

Camm, E. J., Gibbs, M. E., Cock, M. L., Rees, S. M., & Harding, R. (2000). Assessment of learning ability and behaviour in low birthweight lambs following intrauterine growth restriction. *Reproduction, Fertility and Development*, *12*(3–4), 165–172.

Doyle, R. E., Freire, R., Cowling, A., Knott, S. A., & Lee, C. (2014). Performance of sheep in a spatial maze is impeded by negative stimuli. *Applied Animal Behaviour Science*, *151*, 36–42. https://doi.org/10.1016/j.applanim.2013.11.009

Dumont, B., & Petit, M. (1998). Spatial memory of sheep at pasture. *Applied Animal Behaviour Science*. https://doi.org/10.1016/S0168-1591(98)00152-X

Edwards, G. R., Newman, J. A., Parsons, A. J., & Krebs, J. R. (1997). Use of cues by grazing animals to locate food patches: An example with sheep. *Applied Animal Behaviour Science*, *51*(1–2), 59–68. https://doi.org/10.1016/S0168-1591(96)01095-7

Elliot, O., & Scott, J. P. (1965). The analysis of breed differences in maze performance in dogs. *Animal Behaviour*, *13*(1), 5–18. https://doi.org/10.1016/0003-3472(65)90065-5

Erhard, H. W., Boissy, A., Rae, M. T., & Rhind, S. M. (2004). Effects of prenatal undernutrition on emotional reactivity and cognitive flexibility in adult sheep. *Behavioural Brain Research*. https://doi.org/10.1016/j.bbr.2003.08.003

Erhard, H. W., Elston, D. A., & Davidson, G. C. (2006). Habituation and extinction in an approach-avoidance test: An example with sheep. *Applied Animal Behaviour Science*, *99*(1–2), 132–144. https://doi.org/10.1016/j.applanim.2005.10.008

Ferreira, G., Keller, M., Saint-Dizier, H., Perrin, G., & Lévy, F. (2004). Transfer between views of

conspecific faces at different ages or in different orientations by sheep. *Behavioural Processes*. https://doi.org/10.1016/j.beproc.2004.08.005

Hebb DO, W. K. (1946). A method of rating animal intelligence. *The Journal of General Psychology*, *34*, 59–65.

Hernandez, C. E., Harding, J. E., Oliver, M. H., Bloomfield, F. H., Held, S. D. E., & Matthews, L. R. (2009). Effects of litter size, sex and periconceptional ewe nutrition on side preference and cognitive flexibility in the offspring. *Behavioural Brain Research*. https://doi.org/10.1016/j.bbr.2009.05.019

Hirata, M., Tomita, C., & Yamada, K. (2016). Use of a maze test to assess spatial learning and memory in cattle: Can cattle traverse a complex maze? *Applied Animal Behaviour Science*, *180*, 18–25. https://doi.org/10.1016/j.applanim.2016.04.004

Hobbs-Chell, H., King, A. J., Sharratt, H., Haddadi, H., Rudiger, S. R., Hailes, S., … Wilson, A. M. (2012). Data-loggers carried on a harness do not adversely affect sheep locomotion. *Research in Veterinary Science*. https://doi.org/10.1016/j.rvsc.2011.06.007

Hosoi, E., Swift, D. M., Rittenhouse, L. R., & Richards, R. W. (1995). Comparative foraging strategies of sheep and goats in a T-maze apparatus. *Applied Animal Behaviour Science*. https://doi.org/10.1016/0168-1591(95)00572-A

Hunter, D. S., Hazel, S. J., Kind, K. L., Liu, H., Marini, D., Owens, J. A., … Gatford, K. L. (2015). Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep. *Physiology and Behavior*, *139*, 244–253. https://doi.org/10.1016/j.physbeh.2014.11.037

Jansen, J., Bolhuis, J. E., Schouten, W. G. P., Spruijt, B. M., & Wiegant, V. M. (2009). Spatial learning in pigs: Effects of environmental enrichment and individual characteristics on behaviour and performance. *Animal Cognition*, *12*(2), 303–315. https://doi.org/10.1007/s10071-008-0191-y

Johnson, T. B., Stanton, M. E., Goodlett, C. R., & Cudd, T. A. (2012). T-maze learning in weanling lambs. *Developmental Psychobiology*, *54*(8), 785–797. https://doi.org/10.1002/dev.20624

Jolly, R. D., Arthur, D. G., Kay, G. W., & Palmer, D. N. (2002). Neuronal ceroid-lipofuscinosis in Borderdale sheep. *New Zealand Veterinary Journal*, *50*(5), 199–202. https://doi.org/10.1080/00480169.2002.36311

Jolly RD, W. D. (1975). Blindness in South Hampshire sheep: A neuronal ceroid-lipofuscinosis. *New Zealand Veterinary Journal*, *24*(6), 123.

Keith, M. K., Ana, P. D. C., Andrea, E. L., Michael, R. H., & Jon, W. P. (2001). Sheep don't forget a face. *Nature*, *414*(6860), 165. https://doi.org/10.1038/35102669

Kendrick, K. M., Atkins, K., Hinton, M. R., Broad, K. D., Fabre-Nys, C., & Keverne, B. (1995). Facial and vocal discrimination in sheep. *Animal Behaviour*, *49*(6), 1665–1676. https://doi.org/10.1016/0003-3472(95)90088-8

Kilgour, R.; Bruere, A. (1970). Behaviour Patterns in Chromatin- positive Klinefelter ' s Syndrome of Sheep, *225*(Cm), 7–8.

Kilgour, R. (1981). Use of the Hebb-Williams closed-field test to study the learning ability of Jersey cows. *Animal Behaviour*, *29*(3), 850–860. https://doi.org/10.1016/S0003-3472(81)80020-6

Knolle, F., McBride, S. D., Stewart, J. E., Goncalves, R. P., & Morton, A. J. (2017). A stop-signal task for sheep: introduction and validation of a direct measure for the stop-signal reaction time. *Animal Cognition*. https://doi.org/10.1007/s10071-017-1085-7

Lee, C., Colegate, S., & Fisher, A. D. (2006). Development of a maze test and its application to assess spatial learning and memory in Merino sheep. *Applied Animal Behaviour Science*, *96*(1–2), 43–51. https://doi.org/10.1016/j.applanim.2005.06.001

McBride, S. D., Perentos, N., & Morton, A. J. (2016). A mobile, high-throughput semi-automated system for testing cognition in large non-primate animal models of Huntington disease. *Journal of Neuroscience Methods*, *265*, 25–33. https://doi.org/10.1016/J.JNEUMETH.2015.08.025

Mitchell, N. L., Russell, K. N., Wellby, M. P., Wicky, H. E., Schoderboeck, L., Barrell, G. K., … Palmer, D. N. (2018). Longitudinal In Vivo Monitoring of the CNS Demonstrates the Efficacy of Gene Therapy in a Sheep Model of CLN5 Batten Disease. *Molecular Therapy*. https://doi.org/10.1016/j.ymthe.2018.07.015

Mole, S. E., Williams, R. E., & Goebel, H. H. (2005). Correlations between genotype, ultrastructural morphology and clinical phenotype in the neuronal ceroid lipofuscinoses. *Neurogenetics*, *6*(3), 107–126. https://doi.org/10.1007/s10048-005-0218-3

Morton, A. J., & Avanzo, L. (2011). Executive Decision-Making in the Domestic Sheep. *PLoS ONE*, *6*(1), e15752. https://doi.org/10.1371/journal.pone.0015752

Palmer, D. N. (2015). The relevance of the storage of subunit c of ATP synthase in different forms and models of Batten disease (NCLs). *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1852*(10), 2287–2291. https://doi.org/10.1016/j.bbadis.2015.06.014

Peirce, J. W., Leigh, A. E., Dacosta, A. P. C., & Kendrick, K. M. (2001). Human face recognition in sheep: Lack of configurational coding and right hemisphere advantage. *Behavioural Processes*, *55*(1), 13–26. https://doi.org/10.1016/S0376-6357(01)00158-9

Rabinovitch, MS., R. H. (1951). A closed field intelligence test for rats. *Canadian Journal of Psychology*, *5*, 122–128.

Rider, J. A., & Rider, D. L. (1988). Batten disease: past, present, and future. *American Journal of Medica Genetics*, *5*, 21–26.

Russell, K. (2017). *Longitudinal in vivo monitoring of the neuropathology in ovine neuronal ceroid lipofuscinoses*. PhD Thesis. Lincoln University.

Russell, K. N., Mitchell, N. L., Anderson, N. G., Bunt, C. R., Wellby, M. P., Melzer, T. R., … Palmer, D. N. (2018). Computed tomography provides enhanced techniques for longitudinal monitoring of progressive intracranial volume loss associated with regional neurodegeneration in ovine neuronal ceroid lipofuscinoses. *Brain and Behavior*, *8*(9), 1–8. https://doi.org/10.1002/brb3.1096

Sanders, D. N., Kanazono, S., Wininger, F. A., Whiting, R. E. H., Flournoy, C. A., Coates, J. R., … Katz, M. L. (2011). A reversal learning task detects cognitive deficits in a Dachshund model of late-infantile neuronal ceroid lipofuscinosis. *Genes, Brain and Behavior*, *10*(7), 798–804. https://doi.org/10.1111/j.1601-183X.2011.00718.x

Shore, D. I., Stanford, L., MacInnes, W. J., Brown, R. E., & Klein, R. M. (2001). Of mice and men: Virtual Hebb-Williams mazes permit comparison of spatial learning across species. *Cognitive, Affective, & Behavioral Neuroscience*, *1*(1), 83–89. https://doi.org/10.3758/CABN.1.1.83

Taylor, D. B., Brown, W. Y., Price, I. R., & Hinch, G. N. (2010). Training Merino sheep to respond to visual and auditory cues. *Animal Production Science*. https://doi.org/10.1071/AN10003