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Manifestation of neuronal ceroid lipofuscinosis in Australian Merino sheep: Observations on altered behaviour and growth

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ABSTRACT

Neuronal ceroid lipofuscinoses (NCL) is an inherited neurodegenerative disorder in children. Presently there is no effective treatment and the disorder is lethal. NCL occur in a variety of non-human species including sheep, which are recognised as valuable large animal models for NCL. This experiment investigated the progressive postural, behavioural and liveweight changes in NCL-affected lambs, to establish practical, non-invasive biomarkers of disease progression for future preclinical trials in a *CLN6* Merino sheep model. A flock of eight lambs at pasture was studied, with the observer blind to the disorder status. Three genotypes were compared: homozygous affected (NCL; $n=4$), clinically normal heterozygous (Carrier; $n=2$) and homozygous normal (non-carrier control (Normal); $n=2$). Direct observation during daylight and continuous accelerometer measurements over 72 h were used to quantify lamb posture and behaviour in 11 sessions between 26 and 60 weeks of age, conducted at 3-5 week intervals. There was a Genotype (G) × Age (A) interaction ($P=0.001$) for liveweight of the lambs in the experiment, with NCL, Carrier and Normal lambs gaining 11.8, 16.5 and 23.4 kg, respectively, between 26 and 60 weeks of age. G × A interactions were also found for walking behaviour (means for NCL, Carrier and Normal genotype groups at 26 and 60 weeks, were 1.7 and 7.9%, 3.3 and 3.1%, and 2.5 and 1.9% of observations, $P=0.008$) and a composite variable of key behaviours identified in the principal components analysis ($P<0.001$), with mean values for NCL lambs increasing three-fold compared to non-affected lambs as age increased. Similarly, NCL lambs became less responsive to visual and auditory stimuli as they aged. Mean responsiveness scores (out of 3) to visual stimuli for the NCL, Carrier and Normal genotypes at 26 and 60 weeks of age were 2.7 and 1.4, 2.8 and 2.9, and 3.0 and 3.0, respectively (G × A, $P<0.001$). Changes in response to auditory stimuli were similar to visual stimuli. NCL lambs took more ($P=0.015$) steps per 24 h than Carrier and Normal genotype lambs, but there was no G × A interaction. At 26 and 60 weeks of age, respectively, NCL lambs took 2724 and 4121 steps per 24 h, compared to Carrier (1708 and 3105 steps) and Normal genotype lambs (2109 and 3506 steps). NCL lambs also performed less ($P=0.018$) grazing behaviour than Carrier and Normal genotype lambs (66.5, 72.3 and 72.5% of observations for NCL, Carrier and Normal lambs, respectively). A number of behavioural changes identified in the experiment could form the basis for a protocol for monitoring and evaluation of disease progression.

1. Introduction

Neuronal ceroid lipofuscinoses (NCL), also known as Batten Disease, are a group of inherited neurodegenerative disorders characterised by the accumulation of autofluorescent storage bodies, mostly within neurons and retinal cells that lead to progressive brain and retinal atrophy (Mole et al., 2011; Anderson et al., 2013). NCL are mostly recessively inherited, and in humans NCL represent the most common neurodegenerative disorder in children (Schulz et al., 2013). Historically, four main types of NCL were recognised based on the time of onset of disease: infantile, late infantile, juvenile and adult (Zeman, 1976). More recently, different variants of the disease have been classified based on at least 13 disease-causing genes, *CLN1-8,10-14* (Williams and Mole, 2012; Warrier et al., 2013). Although these variants share similarities, they can differ in the age of onset, severity and rate of progression of clinical signs and pathology, and composition and ultrastructure of storage material (Mole et al., 2011). Presently, several underlying

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pathological mechanisms for these diseases are under discussion (Palmer et al., 2013) and several therapeutic approaches are being evaluated in clinical trials, but there is no effective treatment available and all forms of NCL are lethal (Mole et al., 2011).

Mutations responsible for NCL have also been identified in non-human animals, and a number of animal models have been investigated to facilitate research into the disease mechanisms and for development and evaluation of therapeutic strategies for NCL (see review by Bond et al., 2013). While small animal models such as mouse models are very useful in studies on NCL, mice have a less complex brain, and do not demonstrate the severe brain atrophy, intense neuronal loss and retinal degeneration characteristics observed in large animals such as with sheep, or human NCL (Frugier et al., 2008). Therefore, the use of large animal (such as sheep) models is increasing, since the NCL clinical signs more closely resemble those of humans and life expectancy is longer in sheep than mice (Tammen et al., 2006). Sheep breeds affected by NCL have included the South Hampshire, Swedish Landrace, Rambouillet, Merino and Borderdale (Jolly et al., 1980; Järplid and Haltia, 1993; Woods et al., 1994; Cook et al., 2002; Jolly et al., 2002). Two different mutations in the *CLN6* gene are responsible for NCL in Merino and South Hampshire sheep (Tammen et al., 2006), NCL in Swedish Landrace is caused by a mutation in the *CTSD/CLN10* gene (Tyynelä et al., 2000), whereas NCL in Borderdale sheep is caused by a mutation in the *CLN5* gene (Frugier et al., 2008). These ovine diseases represent models for human variants caused by mutations in the corresponding human genes.

The onset of clinical signs of NCL in the *CLN6* Merino sheep model used in this study is reported to occur from 7 to 10 months of age (Tammen et al., 2001, 2006; Cook et al., 2002). Clinical signs include behavioural change, motor deficit, seizure and visual impairment. Reported behavioural changes include reduced awareness of the environment, decreased response to auditory stimuli, startle response to visual and auditory stimuli, aimless walking and walking in circles (Cook et al., 2002; Tammen et al., 2006). The flocking behaviour also appears to diminish and NCL-affected sheep may be found isolated from their group. Motor disturbances observed in NCL-affected sheep include the dragging of feet, stumbling, tremors and at late stage of disease, seizures (Tammen et al., 2006). Profound visual impairment occurs from about 19 months of age, with death by 27 months of age (Cook et al., 2002). The present experiment is the first known controlled experiment investigating the temporal changes to the behavioural time budget and responsiveness of Merino sheep with the homozygous, heterozygous and non-affected genotypes. Previous studies by Tammen et al. (2001, 2006) and Cook et al. (2002) were genetic or prospective clinical-based trials.

The objectives of this experiment were to analyse the progressive postural, behavioural and liveweight changes in NCL-affected Merino sheep compared to heterozygous carriers and homozygous normal sheep, and to establish behavioural indicators that may enable earlier identification of neurological changes in NCL-affected sheep. The findings of the present experiment may assist in measuring the effectiveness of therapeutic trials in sheep, which could potentially assist with trialling therapies for human late infantile NCL.

2. Methods

The experiment was conducted at the University of Sydney Farms, Camden NSW Australia, under approval from the University of Sydney Animal Ethics Committee, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. While the lambs were cared for using standard sheep husbandry management procedures, an additional requirement of the Animal Ethics approval was that all sheep in the flock were inspected daily. This was performed by stock people experienced in the management of NCL-affected sheep observing all sheep at least once daily from a short distance. In addition, the sheep were brought into a yard at least monthly for closer inspection, weighing and individual health checks. The experiment commenced in mid-March and concluded in late November, 2011. As described later, the disease status of all lambs born in this flock was identified using a direct DNA test. Thus all NCL-affected animals were known to the main researchers and stockpeople before onset of clinical signs, which were expected to commence after about 7 months of age. The AEC protocol required that NCL-affected animals were euthanised before severe mental retardation occurred. In extreme cases in the research flock, this had occurred at about 2 years of age, but lambs identified as NCL-affected were nevertheless closely monitored from 6 months of age for any signs of behavioural change, such as standing quietly in the paddock, diminished fear response if the human approached or loss of herding instinct. Similarly, if seizures were observed or if the state of health deteriorated (e.g. it was noticed that the individual had lost weight, was not feeding, drinking or showed signs of physical discomfort, distress or suffering), they were euthanised. The approved method of euthanasia was via barbiturate over dose through intravenous injection of Sodium Pentobarbitone.

2.1. Subjects and experimental procedures

An experimental Merino *CLN6* research flock was maintained at University of Sydney Farms, Camden, which was based on affected and carrier sheep that were sourced from two medium-wool Merino flocks in northern New South Wales (Tammen et al., 2001; Cook et al., 2002). Clinically normal carrier ewes and carrier rams were mated to produce homozygous affected, heterozygous and homozygous normal lambs in an expected ratio of 1:2:1. Thirty-six lambs born during the 2010 lamb-drop were ear tagged for individual identification and subsequently tested for genotype using a direct DNA test for the disease-causing c.184C > T mutation in ovine *CLN6* (Tammen et al., 2006). Four female lambs homozygous for the c.184C > T mutation (i.e. NCL-affected) were selected for the present experiment, and a sub-flock of eight female lambs was formed consisting of the four NCL-affected lambs, two heterozygous (Carrier) and two non-affected (Normal) lambs. The eight lambs had been born over a 2-week period in September–October 2010. Birth weight of the four NCL-affected lambs were 1.7, 2.6, 3.75 and 3.73 kg, while it was 4.15 and 4.44 kg for the Carrier and 4.2 and 6.0 kg for the Normal lambs. The lambs were weaned from their dams at 23.9 (±SD 0.57) weeks of age. The eight experimental lambs were maintained as a single group, alternately grazed in two adjacent paddocks measuring 800 and 840 m^2, respectively, to manage pasture availability and worm burdens. Observation of lamb behaviour commenced 2 weeks after weaning and the experiment proceeded for 8 months. During this time, behaviour observations were conducted in 11 observation weeks, at approximately 3–5 week intervals. All behaviour observations were recorded while lambs were located in the 800 m^2 paddock; the lambs had been in the paddock for at least 4 days prior to commencement of the respective observation week.

On Monday morning in observation weeks the lambs were brought to a nearby yard by two experimenters. Each lamb's ear tag number was recorded and a number between 1 and 8 was sprayed on the sides of the lambs using scourable spray-mark (Leader Stock Marker), for visual identification in the paddock. The respective identifying numbers were allotted at random at the commencement of the experiment, so that the experimenter who conducted the direct visual observations would be blind to the genotype status. A commercially available 3-axis accelerometer (IceTag® Sensor,