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A Molecular Dissection of Neuroinflammation in Ovine Batten Disease

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University

by
Jarol Zhe Chen

Lincoln University
2016
A Molecular Dissection of Neuroinflammation in Ovine Batten Disease

by

Jarol Zhe Chen

Batten disease (Neuronal ceroid lipofuscinosis, NCL) is a group of devastating neurodegenerative diseases that affect children, caused by mutations in a number of genes, but the underlying pathogenic mechanisms remain unclear. However, the remarkable similarity of pathological features between classical forms of NCL indicates that there may be a unique pathway in which NCL proteins play a role. Immunohistochemical investigations of ovine Batten disease revealed neuroinflammation preceded neurodegeneration in a regionally specific manner. A previous study showed that chronic treatment with an anti-inflammatory compound minocycline did not inhibit the neuroinflammation, indicating the need to understand the specific neuroinflammatory cascade in Batten disease to identify likely druggable targets before embarking on drug therapies. A drug screening method is not practical in large animals. Thus, a proposed neuroinflammatory cascade was investigated in a longitudinal study of CLN6 affected sheep brains. This thesis describes the changes in selective neuroinflammatory modulators in this proposed neuroinflammatory cascade during the disease progression.

Selected neuroinflammatory modulators (TNF-α, TGF-β, IL-1β, IL-10, NF-κB, MAPK14, JAK/STAT, SOCS3, MnSOD, iNOS, Trk B and BDNF) were investigated by quantitative PCR on RNA extracted from different brain regions across ages of disease development (2, 6, 9, 18 and 24 months). Longitudinal expression of MnSOD and mitochondrial marker (COX IV) was also studied by immunohistochemistry on perfusion-fixed brain sections.

The distribution of MnSOD through the cortical grey matter co-localised to mitochondria and became compressed to the boundary between layers I and II and between layers IV and V in the affected cortices at 18 and 24 months, following severe neurodegeneration. There was
no such compression in the non-degenerating cerebellum and brain stem. Quantification of MnSOD and COX IV across the cortical grey matter showed that the expressions were reduced at 18 and more so at 24 months, indicating that previous reports of enhanced activity are likely to have arisen from unmatched sampling.

Anti-inflammatory SOCS3 mRNA expression was up-regulated significantly prior to neurodegeneration and concurrent to the neuroinflammation, and was accompanied by up-regulation of its protein expression. It however did not suppress the neuroinflammation. Both pro- and anti-inflammatory cytokines (TNF-α, IL-1β, TGF-β, IL-10) were upregulated at the initiation of neurodegeneration, at 4-6 months of age, prior to the onset of clinical disease and cortical atrophy evident at 10-14 months, whereas the oxidative responsive genes SOD2/MnSOD and HO-1 were not. iNOS expression was not found in sheep. NF-κB, MAPK14 and JAK/STAT pathway activation followed elevation of cytokines. TrkB expression was increased in the advanced disease while BDNF expression remained unchanged. Other recently proposed neuroinflammatory modulators (PI3Ks, MT I/II/III, GRN) were also investigated. The up-regulation of MT I/II/III followed the increase of cytokines and PI3Ks were not changed by the disease. GRN, a mutation in which was thought to cause a new form of NCL, showed no causative role in CLN6.

These results discount the oxidative stress and disruption of blood brain barrier, and reveal an uncontrolled neuroinflammatory pathway mediated by irregular cytokine signalling. This study highlights the weakness of building scenarios based on observed changes in single gene expressions in isolation. Also discussed are the problems associated with the newly assigned NCLs that lack a thorough biochemical analyses. A similar study of CLN5 ovine Batten disease underway will indicate the generality of these findings. A change of strategy is planned to continue these studies. Given the large number of changes observed, a transcriptomic approach is probably more appropriate.

**Note:** Molecules investigated in this study are fully defined in the abbreviation list.

**Keywords:** Batten disease, neuronal ceroid lipofuscinosis, lysosomal storage disorder, animal models, sheep, neuroinflammatory cascade, neurodegeneration, oxidative stress
Declaration

Publications


- Drafts are in preparation for publication.
  - Characterisation of key oxidative stress-responsive genes and gene products in ovine CLN6.
  - A molecular dissection of neuroinflammatory cascade in ovine CLN6.

Presentations


Statement of candidate contribution and significance of this thesis

This thesis was part of a larger-scale project to study neuroinflammation in ovine models of NCL, largely funded by the Australian and American chapters of the Batten Disease Support and Research Association (BDSRA) and supported by a collaborative network, BARN (Batten Animal Research Network) as well as Kathleen Ann Stevens scholarship, MacMillan Brown Agricultural Research scholarship and John W & Carrie McLean Trust scholarship. Travel grants were provided by Maurice & Phyllis Paykel Trust and New Zealand Society for Biochemistry and Molecular Biology and Neurological Foundation of New Zealand. Research
in this thesis has led to a grant in preparation for a transcriptomic study in Batten disease sheep, in a collaboration with a Batten research group led by Dr I Imke, Faculty of Veterinary Science, The University of Sydney. Meanwhile, there were people who laid the foundation to this work. Guidance was provided by supervisors, Prof DN Palmer, NL Mitchell and Assoc Prof J Morton for the entire thesis. Prof DN Palmer, Dr MJ Oswald, Dr G Kay, and Dr LA Barry laid the ground work for establishing a central role of neuroinflammation in Batten disease. Dr G Kay, Dr LA Barry and Dr LF Mao initiated the investigation of MnSOD that supported the observation in this thesis.
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I wish to express my fullest appreciation to the people who helped me at some stage throughout my studies. My associate supervisor, Assoc Prof Jim Morton, helped me with my study and write-up of this thesis. Thanks to him for being considerate and supportive when there were troubles with my studentship. I’d like to thank the staff at JML and Ashley Dene for looking after the research animals. Thanks to Prof Peter Shepherd, Dr Greg Smith and Dr Hayden McEwen, and all the lab members in the Signal Transduction Group, Department of Molecular Medicine and Pathology, Auckland University for providing me technical support and advice on PI3Ks work. Thank you also to Dr Graham Kay, who has been always happy to help out with any problems or requests.

The financial support from the Batten Disease Support and Research Association (BDSRA), John W & Carrie McLean Trust, Kathleen Ann Stevens scholarship and MacMillan Brown Agricultural Research scholarship from Lincoln University have made
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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimers disease</td>
</tr>
<tr>
<td>ANCL</td>
<td>adult NCL</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>BR</td>
<td>bilirubin</td>
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<tr>
<td>BV</td>
<td>biliverdin</td>
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<td>BVR</td>
<td>biliverdin reductase</td>
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<td>CAMs</td>
<td>cell adhesion molecules</td>
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<tr>
<td>CL</td>
<td>curvilinear</td>
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<td>CLN</td>
<td>NCL causing gene</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>COX</td>
<td>cytochrome c oxidase</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>CTSD</td>
<td>cathepsin D</td>
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<td>DAB</td>
<td>3, 3’-diaminobenzadine</td>
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DNA  deoxyribonucleic acid
EDTA  ethylenediaminetetraacetic acid
ER  endoplasmic reticulum
eNOS  endothelial nitric oxide synthase
EM  electron microscopy
FAD  flavin adenine dinucleotide
FERM  F for 4.1 protein, E for ezrin, R for radixin and M for moesin
FMN  flavin mononucleotide
GABA  γ-aminobutyric acid
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GFAP  glial fibrillary acidic protein
GnRH  gonadotrophin-releasing hormone
GRODs  granular osmiophilic deposit
GSB4  *Griffonia simplicifolia* isolecitin type I-B4
HD  Huntingtons disease
HO-1  heme oxygenase-1
HRP  horseradish peroxidase
IFN  interferon
IGF-1  insulin-like growth factor-1
IL  interleukin
INCL  infantile NCL
iNOS  inducible nitric oxide synthase
<table>
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<th>Acronym</th>
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<td>Jak/STAT</td>
<td>janus kinase/signal transducers and activators of transcription</td>
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<td>JNCL</td>
<td>juvenile NCL</td>
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<td>LDS</td>
<td>lithium dodecyl sulphate</td>
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<td>LFA-1</td>
<td>lymphocyte function associated antigen 1</td>
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<tr>
<td>LINCL</td>
<td>late infantile NCL</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LSD</td>
<td>lysosomal storage disease</td>
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<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MPS</td>
<td>mucopolysaccharidosis</td>
</tr>
<tr>
<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NCL</td>
<td>neuronal ceroid lipofuscinoses</td>
</tr>
<tr>
<td>NF</td>
<td>normalisation factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation signal</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>ONOO-</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline, pH 7.4</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline, pH 7.4, containing 0.3% Triton X-100</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinsons disease</td>
</tr>
<tr>
<td>PHOX</td>
<td>phagocytic oxidase</td>
</tr>
<tr>
<td>PPT1</td>
<td>palmitoyl protein thioesterase 1</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>REML</td>
<td>restricted maximum likelihood method</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPLPO</td>
<td>large ribosomal protein PO</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAP</td>
<td>sphingolipid activator proteins</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signalling proteins</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase (gene)</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline, pH 7.4</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TD</td>
<td>transactivation domain</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptors</td>
</tr>
<tr>
<td>TPP1</td>
<td>tripeptidyl peptidase I</td>
</tr>
<tr>
<td>vLINCL</td>
<td>variant late infantile NCL</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review

1.1 Neuronal ceroid lipofuscinosis

Batten disease (neuronal ceroid lipofuscinosis, NCL) is a group of human autosomal recessive neurodegenerative diseases which usually lead to visual failure, seizures, progressive mental and motor deterioration and premature death (Haltia, 2006). The disease frequency has been estimated to be 1 in 12,500 live births worldwide (Rider & Rider, 1988). NCLs are typically classified by the age of onset of clinical symptoms. These vary from newborn to adulthood, but the common forms of NCL usually have an onset age between childhood and adolescence. Affected individuals have a healthy and normal development followed by clinical symptoms of dementia, visual loss, epilepsy and movement disabilities (Anderson et al, 2013). Progression of the clinical symptoms is variable between patients. The neuropathology of the affected individuals contains two key aspects. Firstly, the pathological accumulation of proteins in lysosome-derived organelles, namely storage bodies, occurs in cells in most tissues throughout the body (Goebel et al, 1999; Haltia, 2003; 2006). These ubiquitous storage bodies contain specific protein aggregations and exhibit distinctive ultrastructural patterns under electron microscopy (EM), depending on the form of NCL (Kousi et al, 2011). A second hallmark is neurodegeneration which is most prominent in the cerebral cortex and the retina (Anderson et al, 2013). Glial activation has been suggested to accompany areas of the greatest neuronal loss (Oswald et al, 2005; 2008; Kay et al, 2006; Tyynelä et al, 2004; Anderson et al, 2013).

There are 8 main NCL genes discovered and 4 additional candidate genes have been proposed to cause this group of detrimental diseases (http://www.ucl.ac.uk/ncl/). It is also possible there are yet to be discovered genes that contribute to this group of diseases. In general the functions of the gene products and the underlying mechanisms of the pathogenesis remain obscure.

Traditionally, classification of a subtype of NCLs has been based on the age of onset, clinical symptoms and EM findings (Kousi et al, 2011). Clinical presentations and the ultrastructure of the storage bodies in tissues such as skin, rectum, skeletal muscle and blood lymphocytes, remain the primary means for initial diagnosis (Anderson et al, 2013). However, diagnoses
solely relying on the above methods can be misleading. Different mutations in the same CLN gene can give rise to different clinical phenotypes whereas the mutations in different CLN genes can result in similar ages of onset and clinical symptoms (Kousi et al, 2011). There are also cases in which members of a family had the same genetic mutation but showed varied clinical phenotypes (Williams et al, 2006). Advances in genetic analysis such as the development of next generation sequencing technology have greatly improved the accuracy of the diagnosis of NCLs (Cotman et al, 2015).

The availability of clinical genetic testing and the greater awareness of health professionals about NCLs are beneficial for early diagnosis of the disease, thereby helping the affected families access supportive care. Unfortunately, currently there are no effective treatments for cure or alleviation of the disease. However, the ongoing studies of pathogenesis for suitable targets for pharmacologic intervention and the development of therapies, including enzyme replacement and gene therapies, hold hope for future treatment.

1.2 Classification of NCL and NCL products

NCLs fall under the group of lysosomal storage disorders (LSDs), which represent a group of about 50 genetic disorders caused by deficiencies of lysosomal and some non-lysosomal proteins. These non-lysosomal proteins can reside in the endoplasmic reticulum (ER), the Golgi apparatus or the endosomal pathway. The underlying pathogenesis of LSDs is complicated and remains unclear (Ballabio & Gieselmann, 2009). In classical forms of LSDs, these deficiencies of enzymes lead to an accumulation of their substrates (eg, Pompe disease) or metabolites (eg, cystinosis) in the lysosome, defective transport of lysosomal proteins (eg, mucolipidosis II/III), accumulation of lipids (eg, Niemann-Pick C1 disease), and improper processing of lysosomal proteins (eg. multiple sulphatase deficiency) (Ballabio & Gieselmann, 2009; Dierks et al, 2009; Ruivo et al, 2009; Vanier & Walkley, 2009). Although in some cases accumulated substrates could predict the deficiency of a specific enzyme (eg, Pompe disease and Gaucher disease), in others the stored proteins and the related mutated proteins could not explain a metabolic link, and far more complex mechanisms are involved beyond an enzyme- substrate/substrates relationship. In NCLs, the function of NCL proteins as well as the correlation between the mutated NCL proteins and the unique storage materials are not understood. These stored proteins are either the subunit c of mitochondrial ATP synthase or sphingolipid activator proteins A and D (SAP A and D) in the lysosomes and lysosomal derived organelles.
There have been six basic subtypes characterised in NCLs, including congenital, infantile, late
infantile, variant late infantile, juvenile and adult. Each subtype is caused typically by
particular NCL genes, namely, \( CLN1, 2, 3, 5, 6, 7, 8 \) and \( 10 \) (Table 1.1). Also, novel NCL-
cauing genes have been proposed, being \( CLN4, 11, 13 \) and \( 14 \) (Table 1.1). Disease causing
mutations have been documented in the 13 human (NCL) genes
(http://www.ucl.ac.uk/ncl/mutation.shtml; 2015).

**Table 1.1 Summary of NCL genes, correlated pathology and clinical phenotypes** compiled
from Kousi *et al*, 2011; Bras *et al*, 2012; Cotman *et al*, 2013; Petkau & Leavitt, 2014; Schulz *et
al*, 2013; http://www.ucl.ac.uk/ncl/mutation.shtml

1GROD: granular osmiophilic deposit; CL: curvilinear; FP: fingerprint; RL: rectilinear
2gene products are soluble proteins
3gene products are transmembrane proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Protein Encoded</th>
<th>Protein storage/Ultrastructure</th>
<th>Subtypes</th>
</tr>
</thead>
</table>
| CLN1/PPT1     | 1p32        | Palmitoyl protein thioesterase (PPT1), soluble\(^2\) | Sphingolipid-activator proteins A and D (SAP A and D)
|               |             |                                  | GRODs\(^1\)                 | Classic infantile       |
| CLN2/TPP1     | 11p15       | Tripeptidyl-peptidase I (TPP1), soluble\(^2\) | Subunit c of mitochondrial ATP synthase
|               |             |                                  | CL\(^1\)                    | Classic late-infantile  |
| CLN3          | 16p12       | CLN3, transmembrane\(^3\)        | Subunit c of mitochondrial ATP synthase
|               |             |                                  | FP, CL, RL\(^1\)            | Classic juvenile        |
| CLN4/DNAJC5   | ?           | Cysteine-string protein alpha (CSP\(\alpha\)), soluble\(^2\) | CSP\(\alpha\) self-aggregates?
|               |             |                                  | GRODs\(^1\)                 | Adult                   |
| CLN5          | 13q21.1-q32 | CLN5, soluble\(^3\)              | Subunit c of mitochondrial ATP synthase
|               |             |                                  | CL, FP, RL\(^1\)            | Variant late-infantile  |
| CLN6          | 15q21-q23   | CLN6, transmembrane\(^3\)        | Subunit c of mitochondrial ATP synthase
<p>|               |             |                                  | FP, CL(^1)                | Variant late-infantile  |</p>
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Chromosome Location</th>
<th>Description</th>
<th>Associated Protein/Enzyme</th>
<th>Disease Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLN7/MFSD8</td>
<td>4q28.1-q28.2</td>
<td>CLN7, transmembrane</td>
<td>? RL, FP</td>
<td>Variant late-infantile</td>
</tr>
<tr>
<td>CLN8</td>
<td>8p23</td>
<td>CLN8, transmembrane</td>
<td>? GRODs, CL</td>
<td>Variant late-infantile</td>
</tr>
<tr>
<td>CLN10/CTSD</td>
<td>11p15.5</td>
<td>Cathepsin D, soluble</td>
<td>SAP A and D GRODs</td>
<td>Congenital</td>
</tr>
<tr>
<td>CLN11/GRN</td>
<td>17q21</td>
<td>Progranulin, soluble</td>
<td>TDP-43 FP</td>
<td>Adult?</td>
</tr>
<tr>
<td>CLN12/ATP13A2</td>
<td>? (canine, Belgian family?)</td>
<td>ATPase type 13A2 (ATP13A2), transmembrane</td>
<td>? FP</td>
<td>Juvenile?</td>
</tr>
<tr>
<td>CLN13/CTSF</td>
<td>?</td>
<td>Cathepsin F (CTSF), soluble</td>
<td>? FP</td>
<td>Adult?</td>
</tr>
<tr>
<td>CLN14/KCTD7</td>
<td>?</td>
<td>Pottassium channel tetramerisation domain containing protein type 7 (KCTD7), soluble</td>
<td>? FP, GROD</td>
<td>Infantile?</td>
</tr>
</tbody>
</table>

Two distinctive types of genetic products have been predicted; either soluble proteins (CLN1, CLN2, CLN5, and CTSD/CLN10) or intramembrane (membrane-bound) proteins (CLN3, CLN6, CLN7, and CLN8).

Among thouse NCLs with the soluble proteins, infantile NCL, CLN1 and late infantile NCL, CLN2 encode the soluble lysosomal enzymes palmitoyl protein thioesterase (PPT1) and tripeptidyl peptidase 1 (TPP1), respectively (Vesa et al, 1995; Sleat et al, 1997). A cathepsin D (CTSD/CLN10) mutation was found to underlie an ovine congenital NCL, subsequently described in humans (Tyynelä et al, 1993; Siintola et al, 2006). The CTSD mutations found are associated with reduced enzymatic activity (Steinfeld et al, 2006). CLN5 encodes a soluble lysosomal glycoprotein with unknown function (Sleat et al, 2005, 2006, 2007).

Some NCL associated proteins have been suggested to be membrane-associated. Studies have suggested the CLN3 encoded protein may reside in the late endosomal/lysosomal membranes and the predicted protein functions may associate with membrane trafficking, endocytosis and autophagy as well as regulation of lysosomal pH (Cotman et al, 2002;
Fossale et al., 2004; Cao et al., 2006; Metcalf et al., 2008). Other genes such as CLN6 and CLN8 have been suggested to encode endoplasmic reticulum (ER) associated proteins (Lonka et al., 2000; Gao et al., 2002; Wheeler et al., 2002; Mole et al., 2004; Siintola et al., 2006).

Recent genetic advances led to the identification of new NCL-causing genes, including CLN4, 11, 12, 13 and 14. Affected individuals carry mutations in these genes and show NCL-like inclusions in cells and present clinical symptoms similar to NCLs. Mutations in CLN4/DNAJC5, which encodes cysteine-string protein alpha (CSP-α), a protein highly expressed in brain, are thought to underlie an autosomal dominant adult onset NCL (Nosková et al., 2011). Palmitoylation and membrane trafficking of CSP-α were shown to be affected by the mutations (Nosková et al., 2011). The homozygous mutation of CLN11/GRN has been proposed to cause an adult onset NCL (Smith et al., 2012). The heterozygous mutation of CLN11/GRN causes an autosomal dominant disease, frontotemporal lobar degeneration with ubiquitinated TDP-43 inclusions (Petkau & Leavitt, 2014). Mutations of CLN12 were initially described in a canine case and this gene was later associated with an NCL family from Belgium (Farias et al., 2011; Wohlke et al., 2011; Bras et al., 2012). The protein product of CLN12 is thought to be a P-type transporter of inorganic cations and other substrates that localises on the lysosomal membrane (Park et al., 2011). CTSF/CLN13 encodes cathepsin F and the mutations appear to cause an adult onset NCL (Smith et al., 2012). CLN14/KTCD7 was found to be associated with an infantile-onset NCL (Staropoli et al., 2012).

1.3 Animal models of NCLs

Studies of the brain pathology of human NCLs are limited to autopsy tissue, which is at the terminal stage of the disease and cannot provide a resolution of the disease progression. Although changes of anatomical structure and associated brain functions can be accessed by brain imaging studies, the cellular and molecular changes coupled to the functional changes are not able to be observed by such neuroimaging techniques.

Animal models are valuable tools for the systemic investigation of the underlying molecular mechanisms and the development of therapies. Mouse models for human disease research have contributed greatly to the understanding of various genetic diseases, but also have limitations such as a short life expectancy (Pinnapureddy et al., 2015). A recent study reported the genetics of mouse models correlated poorly with human inflammatory disease (Seok et al., 2013). This study brought forth a discussion regarding the use of mouse models.
for human inflammatory diseases (Cauwels et al, 2013; Osterburg et al, 2013; Tompkins et al, 2013; Warren et al, 2013; Shay et al, 2015; Takao & Miyakawa, 2015; Warren et al 2015). A special issue published by Molecular Immunology has emphasised the benefits from the use of large animal models, as well as the importance of variations between large animals and the mouse in terms of immunogenetics, anatomy, and the mechanisms controlling immune responses (Montoya & Meurens, 2015). The use of multiple animal models for human disease research may be able to compensate for the limitations of mouse models.

NCLs animal models can be naturally occurring or could be created by genetic engineering (Cooper et al, 2006). A number of spontaneous NCL forms have been discovered in animals, including dogs (Koppang, 1970; Jolly et al, 1994), cats (Green & Little, 1974; Bildfell et al, 1995; Weissenbock and Rossel, 1997), sheep (Jolly & West; 1976; Jolly et al, 1994; Tammen et al, 2001; Cook et al, 2002), goats (Fisk & Storts, 1988), cattle (Harper et al, 1988), horses (Url et al, 2001) and mice (Bronson et al, 1993, 1998).

The ovine models of CLN5 and CLN6 have been well established at Lincoln University, New Zealand. They are long lived compared to rodents and therefore suitable for systemic investigation of biochemical changes during disease progression and the effects of treatments. Ovine brains are larger than rodent brains and similar in structure to human brains. The clinical presentation in affected animals resemble those in the human diseases (Bond et al, 2013; Pinnapureddy et al, 2015). Ovine CLN6 disease in New Zealand South Hampshire sheep is the best characterised of all the animal models of NCL (Palmer & Tammen, 2011). The first animal was diagnosed for decades ago and the model has been well characterised since then (Jolly & West, 1976; Graydon & Jolly, 1984; Mayhew et al, 1985; Jolly et al, 1980, 1982, 1988, 1989, 1992; Jolly & Palmer, 1995). These ovine models for NCL have been proved to be informative of the pathogenesis (Bond et al, 2013; Pinnapureddy et al, 2015). The well-documented pathological changes in disease progression of ovine CLN6 yield a better understanding of disease mechanism and allows common hypotheses about NCL neuropathogenesis to be made.

1.4 Common pathology of NCL

1.4.1 Storage bodies

Accumulation of fluorescent storage bodies in neuronal cells, as well as many other cell types in the body, is a hallmark of NCL (Goebel et al, 1999; Haltia, 2003; 2006). In the CNS,
accumulation of the storage material in lysosome derived organelles localise in nerve cell bodies and axonal compartments. As indicated in Table 1.1, the storage bodies have various ultrastructures determined by EM, consisting of GRODs, CL, FP and RL, of which images can be found in various review papers regarding NCL pathology (Anderson et al., 2013). SAP A and D storage seems to correlate predominantly with the GRODs ultrastructure (Anderson et al., 2013).

The subunit c of the mitochondrial ATP synthase was identified to be the main protein pathologically accumulated in CLN2, CLN3, CLN5, CLN6, CLN7 and CLN8 NCL forms while the SAP A and D, which are small heat-stable glycoproteins required for the hydrolysis of sphingolipids in lysosomes, are the main storage materials in the CLN1 and CLN10 forms (Animal models: Fearnley et al., 1990; Martinus et al., 1991; Jolly et al., 1994; Pardos et al., 1994; Palmer et al., 1997; Url et al., 2001; Cook et al., 2002; Katz et al., 2005; Melville et al., 2005; Frugier et al., 2008; Human NCLs: Palmer et al., 1989a, 1989b, 1992; Hall et al., 1991; O’Brien & Kishimoto, 1991; Tyynelä et al., 1993; Kominami et al., 1992; Kida et al., 1993; Tyynelä et al., 1997; Herva et al., 2000; Siintola et al., 2006). The storage of other proteins has been suggested in recently discovered NCLs (Table 1.1), however the biochemical analyses of the protein storage in these forms has not been published.

The storage bodies are strongly fluorescent and have a maximum excitation range between 350nm and 370nm and a maximum emission range from between 490 and 520nm (Palmer, 1987), which is similar to the fluorescent spectra of the material generated in vitro by reacting protein and peroxidised lipids (Chio et al., 1969a, b). Hence, the fluorescence of the storage bodies was postulated to be derived from the products of lipid peroxidation. However, in spite of the “autofluorescent” property of storage bodies, the storage bodies do not contain fluorophors and no evidence of lipid peroxidation was found (Palmer et al., 1985, 1986b, 1988, 1993, 2002; Hall et al., 1989). The fluorescent property may arise from light diffracting from the arrangement of the protein aggregates in the storage bodies, due to the highly hydrophobic nature of subunit c (Palmer et al., 2002).

A genetic and a metabolic defect, such as a defective protease were proposed to result in the accumulation of subunit c, but these hypothesis were eliminated by characterisation of subunit c. The nuclear genes coding for subunit c, P1, P2 and P3 as well as their expression were normal in affected sheep (Medd et al., 1993). Mass spectrometry analysis of
accumulated subunit c has shown that the complete and normal subunit c is stored (Chen et al, 2004). The normal post-translational modifications including trimethylation of lysine-43 and cleavage of the lead sequence indicate that the stored subunit c has been processed into mitochondria prior to its accumulation in lysosomes (Palmer, 2015; Walpole et al, 2015). Also, the accumulation of subunit c caused by a lack of TPP1, a lysosomal protease which may be involved in subunit c degradation in LINCL/CLN2, is not able to explain the commonality of subunit c accumulation in various forms (Sleat et al, 1997; Ezaki et al, 1999; Warburton & Bernardini, 2000; Xu et al, 2010).

Any relationship between the storage material accumulation and subsequent cell death remains unclear. In spite of the storage material being ubiquitous, cell death appears to be specific to a sub-population of neurons in the CNS and neural retina (Oswald et al, 2005, 2008; Cooper et al, 2006; Anderson et al, 2013). In the ovine model of CLN6, the accumulation of storage bodies was evident throughout the brain of CLN6 affected sheep as early as 12 days after birth and became more prominent with age. This process was neither coupled with glial activation nor neurodegeneration (Oswald et al, 2005). Many cells which accumulate storage bodies, such as glial cells, Purkinje cells in cerebellum and normal ventral horn cells of the spinal cord of affected lambs, are not afflicted by neurodegeneration (Jolly et al, 1989; Palmer et al, 2002; Oswald et al, 2005). There is also massive storage in many visceral tissues such as liver which are otherwise unaffected. Thus, storage body accumulation is a separate manifestation of the NCL genetic lesions. However, the similar composition of the storage bodies suggests disruptions in a common biological pathway, in which all NCLs proteins play a part, that may involve subunit c of mitochondrial ATP synthase trafficking or degradation (Palmer, 2015).

1.4.2 Neurodegeneration

Neurodegeneration is a prominent feature in NCLs and correlates with the disease progression and the onset of clinical symptoms. The atrophy of the brain occurs predominantly in the cerebral cortex and occasionally in the cerebellum. Subcortical nuclei are relatively preserved, although hippocampal involvement has been reported (Tyynelä et al, 2004; Tokola et al, 2014). Atrophied white matter and increases in cerebrospinal fluid-carrying spaces and the ventricles have also been described (Anderson et al, 2013). Retinal degeneration is also a prominent feature noted in NCLs, CLN1, 2, 3, 5, 6, 7 and 8 (Anderson et al, 2013).
Progression of neurodegeneration is dependent on the species, the form of NCL and the type of mutation. In ovine CLN6, degeneration progresses in a regionally specific manner initially evident in the visual cortex, followed by the parieto-occipital and somatosensory cortices and progressed to the motor cortex. The degeneration affects the entire cortical mantle in advanced near terminal disease. The cerebellum and the subcortical nuclei were not affected by the degenerative events in ovine NCL, but cerebellar atrophy occurs in some human forms (Oswald et al., 2005; 2008; Goebel & Wisniewski, 2004). There are differences between the murine and ovine models and humans in the regional vulnerability of neurons. Murine models display vulnerability of the sensory thalamocortical pathways while sheep and humans show vulnerability of neurons in the cortex and hippocampus (Palmer et al., 2013).

Depletion of the neurons as well as the selective degeneration of neurons underlies the progressive regional atrophy. Retinal degeneration occurs primarily in the photoreceptor cells and bipolar neurons of the outer nuclear layer, and is less dramatic in other layers of retina, whereas the pigment epithelium is relatively well preserved (Graydon & Jolly, 1984). Braak and Goebel (1978) reported selective degeneration of the small stellate cells in cerebral cortex. Subsequent studies revealed that the pyramidal neurons in cerebral cortical layers II to V are vulnerable to neurodegeneration; the precise course depending on the form of NCL (Jolly, 1995; Herva et al., 2000; Haltia, 2003; Oswald et al., 2005). In ovine CLN6, Nissl staining showed that the neurons between the cerebral cortical layers II to III and V to VI are most vulnerable to degeneration, especially the pyramidal neurons in layer III, while those in layer V are better preserved (Oswald, 2004).

The selective degeneration of GABAergic interneurons has been described in human NCL patients and animal models of NCLs (Braak & Goebel, 1978; 1979; Cooper et al., 1999; 2003; Bible et al., 2004; Pontikis et al., 2004; Kielar et al., 2007; Oswald et al., 2001; 2008; Kay et al., 2011). In animal models of NCLs, the complex patterns of degeneration in the interneuron populations differ between locations (Bible et al., 2004; Pontikis et al., 2004; Kielar et al., 2007; Oswald et al., 2001; 2008; Kay et al., 2011), suggesting that the cellular location and connectivity are the determinants of neuron survival (Oswald et al., 2008). The underlying molecular cues and the susceptibility of neurons to such molecular cues may determine the neuronal fate.
1.4.3 Neuroinflammation

Localised glial activation has been found in various models of NCL (Bible et al, 2004; Pontikis et al, 2004; Oswald et al, 2005; 2008; Kay et al, 2006; Kielar et al, 2007; Partanen et al, 2008; Macauley et al, 2011; Kuronen et al, 2012). Glial activation precedes neuronal depletion in a regional manner, suggesting that neuroinflammation plays a central role in pathogenesis and is a predictor of neuronal loss (Palmer et al, 2013). Immunohistochemical studies in ovine CLN6 show glial activation had begun in perinatal sheep, proceeded regionally, and preceded neurodegeneration, firstly affecting the visual cortex, followed by the parieto-occipital and somatosensory cortices and subsequently the motor cortex and the entire cortical mantle (Oswald et al, 2005; 2008; Kay et al, 2006; 2011). In concurrent studies, losses from the interneuronal population in ovine CLN6 was characterised as progressive, selective, regional and cell- type specific (Oswald et al, 2008; Kay et al, 2011). Glial activation occurs prior to the neuronal loss and mirrors a successive neuronal loss in the different cortical regions, indicating glial activation plays an essential role in the NCL pathogenesis.

The underlying mechanism of neuroinflammation and how it results in neuronal loss is not understood. Glial activation could either be a stress response to dysfunction of neurons or a cause of neuronal depletion (Oswald et al, 2005; Palmer et al, 2013). Genetic defects of NCLs may also cause intrinsic defects in glial cells, which lead to failure to attenuate neuronal degeneration. In a murine model of amyotrophic lateral sclerosis (ALS), the chimeric mice that are mixtures of normal and SOD1 mutant-expressing cells showed that reduced microglial expression of mutant SOD1 could result in a prolonged life expectancy and a delay in the disease progression (Clement et al, 2003; Boillé et al, 2006).

Glial cells are both the primary target and the source of cytokines. Cytokines are a group of signalling molecules that are generally implicated in the regulation of the immune response and cell survival. They are generally expressed at very low levels in healthy individuals and are induced rapidly in response to injury, infection or inflammation. Up-regulation of cytokine-related genes was reported in a mouse model of INCL (Qiao et al, 2007). A systemic study on the expression profile of pro- and anti- inflammatory cytokines in ovine CLN6 revealed chronic up-regulation of pro- (Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β)) and anti- inflammatory (Transforming growth factor- β (TGF-β), Interleukin-10 (IL-10)) cytokines in presymptomatic affected animals (Barry, 2011). The efficacy of CNS directed gene
therapy has been investigated in murine INCL, in conjunction with an anti-neuroinflammatory drug targeting glial activation. The combined treatment dampened glial activation, reduced brain atrophy, and extended the life span (Macauley et al., 2014). These results show that treatment with a mechanism-based anti-inflammatory drug as an adjunct to gene therapy may have potential for treating NCLs.

1.5 CLN6

It is difficult to obtain human tissues for research on the successive stages of the disease progression. Many aspects are involved such as the low incidence of the disease, human ethics, and the requirement for non-invasive methods and stringent criteria for drug trials. Animal models allow direct access to all tissues throughout the disease course, which can be monitored from the early developmental stages up to death. Among all the animal models for NCLs, ovine CLN6 is a well-established model that has a great value for studying human disease and laid the foundation of the studies in this thesis.

1.5.1 Human CLN6

CLN6 causes a variant of late infantile NCL, with a slightly older onset age than CLN2, between 3 to 8 years (Kousi et al., 2011). The CLN6 form of NCL has been found to affect different populations around the world, including people in the Americas, South and Western Asia, North Africa, and Europe (http://www.ucl.ac.uk/ncl/CLN6patienttable.htm). The ultrastructures of storage materials are primarily mixed curvilinear and fingerprint (Mole et al., 2005). Though mutations of CLN6 show variation in the age of onset and disease progression, the patients present with early seizures and motor difficulties followed by visual impairment (Mole et al., 2005). CLN6 has also been reported to associate with adult onset Kufs disease type A (Arsov et al., 2011). The onset age of the CLN6 related Kufs disease is between teenage years (16yrs) and the middle age (50yrs). Kufs disease type A caused by mutated CLN6 differs from variant late infantile NCL in that there is a much later age of onset and a lack of visual impairment (Arsov et al., 2011).

The CLN6 gene is located on chromosome 15q21-23 in humans and is predicted to encode a novel 311 amino acid membrane bound protein with two ER retention signals (Wheeler et al., 2002; Gao et al., 2002; Mole et al., 2004; Heine et al., 2007). Mutations of CLN6 including 38 missense, five nonsense, five splice-site, one deletion-insertion, four insertion, and 10 deletions have been found in humans (Kousi et al., 2011). The CLN6 protein function is not
understood and how the mutations lead to lysosomal dysfunction is unclear, though theories have been proposed. For instance, the product of some missense mutations in the predicted transmembrane domains of CLN6 were thought to be recognised at the ER and subject to rapid degradation in a proteasome dependent manner thus leading to a loss of the cellular function in the downstream pathway (Oresic et al, 2009). A lack of reliable CLN6 antibodies limits the study of CLN6 protein localisation and its function.

1.5.2 Ovine CLN6

Two ovine models for CLN6 were discovered, one in South Hampshire sheep in New Zealand and the other in Merino sheep in Australia. Ovine CLN6 localises on OAR7 (ovine chromosome 7). The coding sequence is 933 bp long and comprises 7 exons (Tammen et al, 2006). Its gene structure is is identical to the human gene and it has 90% sequence homology to human CLN6.

The causative mutation in ovine CLN6 comprises a 402bp deletion and a 1bp insertion and includes the 83bp sequence of the complete first exon of ovine CLN6 in South Hampshire sheep. This deletion contains one of the highly conserved methionines, which may act as a start codons (Ismail, 2014). It is unlikely any whole mRNA could be made in affected animals. However, there is evidence that expression of mRNA fragments was reduced in CLN6 affected animals, suggesting alternative splicing variants may be present (Tammen et al, 2006; Ismail, 2014). The reduction of mRNA has also been reported in the nclf mouse strain which is a murine model of CLN6. The affected mouse has the insertion mutation in exon 4 in Cln6 identical to late infantile patients from Costa Rica (Gao et al, 2002; Wheeler et al, 2002; Thelen et al, 2012).

A neutral allelic variant c.822G>A found in the coding region of ovine CLN6 is 111 bases downstream from the 5’ end of exon 7 and is the third base of a triplet coding for alanine in affected South Hampshire sheep. This variation alters a restriction site for the enzyme Hae II. The CLN6 affected animals exclusively carry only the A allele (Tammen et al, 2006). This flock of sheep is constructed so that all non-affected CLN6 animals carry the G allele, making the A/G dichotomy disease-linked. By this manipulation, the enzyme digestion of the ovine CLN6 product yields different PCR fragments and is routinely used as a diagnostic tool (Fig 2.1 in Chapter 2).
A different mutation, c.184C>T in exon 2, was found to cause a major amino acid exchange (p.Arg62Cys) in affected Merino sheep and potentially results in a non-functional protein (Tammen et al, 2006). The two distinctively different types of mutations in CLN6 lead to a very similar pathological presentation with slightly different disease progression between South Hampshires and Merinos (Cook et al, 2002; Personal communications, Imke Tammen & Nadia Mitchell, 2015). These are suitable models for human disease, and allow study of the impact of different mutations in the same gene could have on the molecular pathology.

Brains of the CLN6 affected South Hampthires show normal growth from birth to 4 months followed by a slow decline (Fig 1.1). The affected animals rarely survive more than 2 years naturally (as opposed to a normal sheep potential lifespan of 8 to 10 years at least) and are usually euthanised before death for humane reasons. Clinical symptoms become apparent at 9-12 months, with impaired vision and neurological disturbances and include involuntary movements, and circling and confusion (Jolly et al, 1980, Jolly et al, 1982). Pathologies are consistent to an NCL, including storage body accumulation in most cell types, neuroinflammation, selective neuronal vulnerability and brain atrophy (Jolly et al, 1980, Jolly et al, 1982; Mayhew et al, 1985; Oswald et al 2005; 2008). Gross pathology changes are restricted to the cerebrum, the size of the affected cerebrum reducing with a thinning of the gyri, accompanied by cranial thickening (Jolly et al, 1980, Jolly et al, 1982). The reduction of the cerebral cortex occurred progressively and regionally as described before (Section 1.4.3). Thinning of the white matter tracts occurs with age and becomes noticeable in the corpus callosum adjacent to the lateral ventricle at 19 months (Jolly et al, 1980, Jolly et al, 1982; Oswald et al, 2005).
Brain atrophy in ovine CLN6 reflects the development of the clinical symptoms. Blindness is the most notable clinical feature and is accompanied by occipital cortex atrophy and loss of photoreceptors in the retina (Jolly et al., 1989; Mayhew et al., 1985). Accumulation of storage material is evident as early as 12 days of age and increases with age throughout the brain (section 1.3.1; Oswald et al., 2005). The ultrastructure of the storage bodies demonstrate a mixture of appearances including multi-lamellar, finger-print and curvilinear profiles (Palmer & Tammen, 2011). The analyses of storage materials of ovine CLN6 showed specific storage of subunit c of mitochondrial ATP synthase (Palmer et al., 1986a, 1986b, 1989; Fearnley et al., 1990). However, the storage of subunit c in most cell types cannot explain the selective and regional neuronal loss. In contrast, the regional glial activation accurately predicts regional cell loss which follows several months later (Oswald et al., 2005; Kay et al., 2006).

Glial activation in the CLN6 affected ovine brains has been observed before birth and suggests an early onset of pathogenesis during brain development (Kay et al., 2006).
presence of reactive astrocytes and the hypertrophy and proliferation of perivascular cells were noted within the developing white matter of the cerebral cortex 40 days before birth. Astrocytic activation was evident within the cortical grey matter 20 days before birth, which was still confined to the superficial laminae 12 days after birth. Clusters of activated microglia were detected in upper neocortical grey matter laminae shortly after birth. The glial activation was initially restricted to individual layers and specific cortical regions but subsequently spread to deeper layers and more widespread cortical and subcortical regions. This process precedes neurodegeneration, which followed the same regional pattern as the glial activation.

The timepoints for the described pathological features and clinical symptoms were used as a guideline for selecting the ages of tissues used in this thesis (Chapter 2).

1.6 Proposed mechanisms of pathogenesis in NCLs

Glial activation is common to many neurodegenerative diseases including all the NCLs (Cooper, 2010; Palmer et al, 2013). Chronic neuroinflammation has been shown to correlate with neurodegeneration (Raichich et al, 1999; Stoll et al, 1999; Neumann, 2001; Streit et al, 2004; Oswald et al, 2005; Eikelenboom et al, 2006; Kim & Joh, 2006). Glial activation precedes neurodegeneration in a progressive and regional manner in ovine CLN6, indicating neuroinflammation is a better indicator of neurodegeneration than the pattern of storage body accumulation (Oswald et al, 2005; Kay et al, 2006). Similarly, selective vulnerability of interneurons has also been reported to follow the pattern of glial activation (Oswald et al, 2008).

Treatment with anti-inflammatory minocycline has shown efficacy against neuroinflammation in rodent model trials, relating to hypoxic-ischemic brain injury, blast-induced traumatic brain injury, Alzheimers disease (AD) and Huntingtons disease (HD) (Ferretti et al, 2012; Kalonia et al, 2012; Kovesdi et al, 2012; Reinebrant et al, 2012). However, minocycline failed to show efficacy against neuroinflammation in HD patients and in ovine NCL (Schwarz et al, 2010; Kay & Palmer, 2013). These results suggest that the chronic inflammatory process is difficult to subdue or that drugs targeted to the critical steps are required. A previous study by Oswald et al, (2008) demonstrated contrasting patterns of GABAergic interneuron loss in different brain regions. Moreover, the specific loss of gonadotrophin-releasing hormone (GnRH) secreting neurons of the hypothalamus, occurred
in the absence of glial activation or storage body accumulation (Kay et al, 2011). Taken together, these findings show that the underlying molecular mechanism that causes and drives glial activation is complex. The regional functionality and connectivity could have an influence on the neuroinflammatory cascade. Therefore, a detailed understanding of the molecular responses in different cortical regions in the affected brains over time is required to determine targets for anti-inflammatory drugs.

The uniform neuropathological features of the NCLs suggest a common final pathogenic pathway that may converge in all the NCL forms. The conundrums of the neuropathogenesis of NCLs are the general defect (the ubiquitous accumulation of storage bodies in the brain) opposed to the regional and selective effect (the regional and laminar activation of glial activation and the selective vulnerability of neurons). The glial activation coupled with the neurodegeneration in discrete cortical regions indicates neuroinflammation is directly associated with the neuronal loss. The chronic neuroinflammation could lead to a significant increase in pro-inflammatory cytokines, oxidative stress, and disruption of the blood brain barrier (BBB) that ultimately cause depletion of the vulnerable neurons.

Oxidative stress remains a hypothesis that leads to the storage bodies in NCLs (Sehafer & Pearce, 2006). It has been proposed to be involved in pathogenesis of the NCLs by rendering neurons more vulnerable to injury and cell death (Kim et al, 2006b; Wei et al, 2008; Vidal-Donet et al, 2013). Mechanisms have been described to cause this oxidative stress, including ER stress (Kim et al, 2006b; Wei et al, 2008), autophagy disturbances (Vidal-Donet et al, 2013), mitochondrial abnormalities (Majander et al, 1995; Dawson et al, 1996; Siakotos et al, 1998) and lysosomal dysfunction (Prasad et al, 1996). Most of these studies were conducted in vitro, which is unreliable for the study of endogenous oxidative stress (Halliwell, 2014). Studies of an appropriate endogenous marker such as manganese superoxide dismutase (MnSOD) as an indicator of oxidative stress would likely be much more informative.

1.6.1 Neuroinflammation and oxidative stress

Microglial activation has been proposed to be accompanied by the activation of phagocytic oxidase (PHOX), which was suggested to be induced by pro-inflammatory cytokines or microglial phagocytosis. The roles of PHOX in microglia are thought to be associated with microglial proliferation, microglial production of pro-inflammatory cytokines and induction of inducible nitric oxide synthase (iNOS) expression. Activated PHOX produces high levels of
the superoxide anions (O$_2^-$) extracellularly, which either dismutate to hydrogen peroxide (H$_2$O$_2$) by extracellular superoxide dismutase or react with nitric oxide (NO) to produce peroxynitrite (ONOO$^-$). Dual activation of PHOX and iNOS in microglial cells upon activation is suggested to be a mechanism of oxidative stress caused by microglial activation (Brown & Neher, 2010).

NO is not a reactive oxidant. It is a highly diffusible gas resulting from the breakdown of arginine to citrulline, catalysed by nitric oxide synthases. Inducible nitric oxide synthase (iNOS) is thought to be an induced form of nitric oxide synthase and proposed to be responsible for ‘pathological’ NO release. The pathological effect of NO has been proposed to be related to its inhibition of cytochrome c oxidase (complex IV) of the electron transport chain and the formation of ONOO$^-$ with O$_2^-$. Cytochrome oxidase inhibition subsequently results in a change of mitochondrial membrane potential, inhibition of mitochondrial ATP synthase and depletion of ATP, accumulation of H$_2$O$_2$, the increased formation of ONOO$^-$ and depletion of other anti-oxidation moieties such as glutathione (Moncada & Erusalimsky, 2002). All of above gives rise to disruptions of calcium (Ca$^{2+}$) homeostasis, opening of the mitochondrial permeability transition pore and the release of apoptotic signals, including cytochrome c and pro-caspases (Petronilli et al., 1994; Moncada & Erusalimsky, 2002).

1.6.2 Oxidative stress

Redox homeostasis is finely tuned by redox proteins in cells. In mitochondria, the concentration of O$_2^-$ is controlled by three different mechanisms (Turrens, 2003): 1) superoxide dismutase; 2) cytochrome c that oxidises O$_2^-$ to regenerate oxygen; 3) O$_2^-$ dismutation facilitated by lower pHs in the intermembrane space. To elaborate, O$_2^-$ leaks from oxidative phosphorylation undergo a O$_2^-$ dismutation to produce H$_2$O$_2$ by intracellular and extracellular dismutases. Hydrogen peroxide is subsequently reduced to water by a range of peroxide metabolising enzymes including catalases, glutathione peroxidases, and peroxiredoxins to maintain submicromolar concentrations (Fridovich, 1983; Winterbourn & Hampton, 2008).

Theoretically, oxidative stress involves unbalanced redox homeostasis caused by elevated reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation, nitrosylation and damage to nucleic acids and eventually cell death. In brief, protein oxidation occurs to enzymes that either possess reactive sites which contains reduced transition metals, or to
thiols (cysteine methionine) and proteins which possess amino acids like lysine, arginine, proline, and threonine that oxidise to form carbonyl groups. Peroxynitrite, generated from the interaction of NO and $\text{O}_2^-$, causes nitrosylation of tyrosine residues and oxidative modification of amino acid residues, including cysteine, tryptophan, methione, and phenylalanine (Shacter, 2000). For DNA damage, hydroxyl radicals (•OH) react with DNA by addition to double bonds of DNA bases, abstraction of H atoms from the methyl groups of thymine and each of the C-H bonds of 2’-deoxyribose, resulting in C$_5$-OH and C$_6$-OH radicals and the allyl radical. These radicals lead to a series of oxidations of nucleotides (Cooke et al, 2003).

Lipid peroxidation is initiated by a hydroxyl radical that attacks a hydrogen from a methylene carbon on an unsaturated lipid by removing the hydrogen and leaving an unpaired electron on the carbon atom to which the hydrogen was originally attached. The “lipid radical” reacts with oxygen and gives rise to a lipid peroxy radical, which is active to abstract a hydrogen from neighbouring methylene of the fatty acid side chain and so on, thus continuing to produce more peroxy radicals that carry on the same peroxidation reaction. This reaction is termed the chain reaction of lipid peroxidation (Halliwell & Chirico, 1993). It is proposed that the peroxidised lipid could react with proteins to give rise to the “fluorophore”. Lipid peroxidation was proposed to cause accumulation of the storage bodies and pathogenesis in NCLs because of their lipofuscin-like fluorescence, which was thought arise from the products of lipid peroxidation (Chio & Tappel, 1969a; b). The mechanism of lipid peroxidation would require the loss of polyunsaturated fatty acids to lipid peroxidation. The lipid peroxidation hypothesis was soon disproved, following the development of techniques to isolate storage bodies and the identification of the major protein component in the storage materials. This is discussed in detail in Chapter 3.

The brain is thought to be vulnerable to oxidative stress because of its high metabolic rate and a lack of capacity of regeneration (Andersen, 2004). The increased oxidative stress was thought to cause mitochondrial dysfunction and glutamate excitotoxicity in neurons (Andersen, 2004; Beal, 2005; Spalloni et al, 2013; Ong et al, 2013; Van Laar et al, 2015).

1.6.2.1 Mitochondrial dysfunction and oxidative stress

One of the major ROS producing cell organelles is the mitochondrion, which also produces antioxidant enzymes, such as MnSOD, to reduce the potential harm of oxidative stress.
(Fridovich, 1983; Sayre et al., 1999). Malfunction of mitochondria is proposed to cause a reduction in ATP production and calcium buffering and to increase ROS production (Beal, 2005). Changes of activities of antioxidant enzymes, as a result of mitochondrial dysfunction, have been described in Alzheimers (AD) and Parkinsons disease (PD) (Zemlan et al., 1989; Sofic et al., 1992; De Leo et al., 1998; Choi et al., 2005). It is postulated that prolonged mitochondrial dysfunction triggers apoptosis via the opening of mitochondrial permeability transition pore (mPTP) when abnormal oxidative phosphorylation and an increase in intracellular Ca\(^{2+}\) takes place, in turn resulting in the release of apoptotic signals such as cytochrome c and activation of caspases-9 and -3 (Lemasters et al., 1998; Duchen, 2000; Crompton, 2004).

### 1.6.2.2 Glutamate excitotoxicity and oxidative stress

Mitochondrial dysfunction has been thought to be involved in the excitotoxic cascade triggered by glutamate. Glutamate excitotoxicity induced neuronal dysfunction and degeneration has been hypothesised to play a role in the pathogenesis of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), AD and PD (Spalloni et al., 2013; Ong et al., 2013; Van Laar et al., 2015). Glutamate is a major excitatory neurotransmitter in the brain. It is released from vesicles in presynaptic terminals by a calcium dependent mechanism to produce an excitatory post-synaptic potential, which is involved in the activation of receptors for long term potentiation (Meldrum, 2000). Glutamate action is terminated by uptake systems located at the synapse and in the astrocytes surrounding the synapses. Glutamate can be converted to glutamine in astrocytes then released into the extracellular space. Glutamine is then picked up by neurons and converted back to glutamate, which is packed into synaptic vesicles by the vesicular glutamate transporters (Spalloni et al., 2013). Glutamate excitotoxicity means over activation of NMDA/AMPA receptors by excessive glutamate, allowing a large Ca\(^{2+}\) influx into cells and leading to intracellular Ca\(^{2+}\) accumulation (White & Reynold, 1996; Spalloni et al., 2013). In part, the increased intracellular Ca\(^{2+}\) causes enhanced metabolic stress on mitochondria and results in mitochondrial membrane depolarization and opening of the mPTP, ROS production and caspase activation (White & Reynold, 1996). Studies of murine NCLs suggested excitotoxicity as part of the pathogenesis of NCLs (Griffin et al., 2002; Kovács et al., 2006; Kovács & Pearce, 2008). There were no disease related glutamate concentration changes in CSF in ovine NCL, indicating glutamate/glutamine cycling is not a defect in all NCLs (Kay et al., 2009). Thus excitotoxicity is unlikely to be a common cause of neurodegeneration in all NCLs.
1.6.3 Study of oxidative stress in vitro

Some free radicals are extremely short-lived, with half-lives of $1 \times 10^6$ seconds for $O_2^-$ and $1 \times 10^{-9}$ seconds for $\bullet$OH (Voet et al, 2006). This makes detection of specific ROS difficult. Formation of ROS may be monitored by such methods as fluorometry and spectrophotometry, chemiluminescence detection and electron paramagnetic resonance. These methods rely on the redox properties of specific ROS but if other species or probes share similar reactivity, they could produce false results (Turrell, 2003). Taking chemiluminescence for example, luminol could be oxidised to make $O_2^-$. Labelling oxidised lipids, protein and DNA using markers in post mortem tissue may be unreliable since the tissue is handled, processed and stored in rich atmospheric $O_2$.

Cell culture studies for oxidative stress are not ideal since cells are isolated from a biological matrix, where the complex interactions of multiple cell types take place. The cells are subjected to “culture shock” when they are exposed in an unnatural biochemical environment and significantly higher-than-normal oxygen concentrations, all of which lead to impairment of cellular anti-oxidative enzymes and increased generation of ROS (Halliwell, 2014). Hence, it is more reliable to detect the change of endogenous oxidative responsive mediators for signs of oxidative stress.

1.6.4 Oxidative stress responsive mediators

Oxidative stress induces activation of cellular defensive mechanisms such as increased MnSOD and HO-1 (Andersen, 2004; Schipper et al, 2009b). In activated microglia, iNOS seems to be the major source of “pathological” NO (Andersen, 2004; Pannu & Singh, 2006).

1.6.4.1 Manganese superoxide dismutase (MnSOD)

A mitochondrial resident protein, MnSOD, converts superoxide anions from the electron transport chain to hydrogen peroxide, which is then converted into water by catalases and glutathione peroxidases (Fridovich, 1983; Sayre et al, 1999). MnSOD is synthesised as a precursor polypeptide encoded by a nuclear gene and imported into the mitochondrial matrix. The import of the MnSOD precursor from the cytosol through the mitochondrial membrane into the matrix is facilitated by two transporters essential for cleavage of the precursor and insertion of manganese (Luk & Culotta, 2001; Luk et al, 2003; Yang et al, 2006). The MnSOD protein is a homotetramer that comprises of two symmetrical four-helix bundles and four C terminal $\alpha/\beta$ domains. Each tetramer subunit is made up of an $\alpha$-helical
N-terminal domain and a mixed α/β terminal domain. The metal binding site lies on the interface of the two domains (Fig 1.2; Perry et al, 2010). The metal ion is stabilised by four metal ligands and a buried solvent (polar) molecule, forming the active site (Fig 1.2). Other residues arising from outside the active centre (Fig 1.2) facilitate the stability of the active centre and the resultant structure is important for the catalytic function of the enzyme (Yamakura et al, 1995; Whittaker & Whittaker, 1997).

**Figure 1.2** Three dimensional structure of human MnSOD. (A) The wild-type homotetrameric MnSOD structure (http://www.rcsb.org/pdb/explore.do?structureId=1LUV), with the four separate polypeptide chains coloured cyan, blue, green and yellow, and the active site manganese ions depicted as purple spheres. (B) The central active site of MnSOD is on the interface of the two domains of each subunit. The four metal binding ligands in the active site (His26, His74, His163, and Asp159) are shown to bind to manganese (Mn) and form a central active sphere in conjunction with a solvent molecule. A hydrogen-bonding network extends from the metal-bound sphere. Two outer-sphere residues, Gln143 and Tyr34, are also linked to the active centre by the key hydrogen bond. A conserved water molecule then mediates the hydrogen bond between Tyr34 and His30, and the latter also forms a hydrogen bond with Tyr166 from an adjacent subunit (Perry et al, 2010).
The catalysis of MnSOD takes place through a redox process in which the metal cycles between oxidised and reduced states:

\[
\text{Mn}^{3+} + \text{O}_2^- \rightarrow \text{Mn}^{2+} + \text{O}_2
\]

\[
\text{Mn}^{2+} + \text{O}_2^- \rightarrow \text{Mn}^{3+} + \text{H}_2\text{O}_2
\]

**1.6.4.2 Inducible nitric oxide synthase (iNOS)**

Endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and iNOS are responsible for the conversion of L-arginine to L-citrulline and NO, in the presence of several co-factors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH4) (Mattila & Thomas, 2014). The catalytic activities of constitutively expressed eNOS and nNOS are calcium dependent and yield a low concentration of NO that serves as a signalling molecule and vasodilator (Griffith & Stuehr, 1995; Garthwaite & Boulton, 1995; Mattila & Thomas, 2014). On the other hand, NO produced by iNOS in cells such as neutrophils and macrophages, has been postulated to play roles in activities against pathogens and suppression of proliferating cancer cells (Babior *et al*, 1973; MacMicking *et al*, 1997; Xu *et al*, 2002). These activities of NO and the expression of iNOS appear to be mainly established in rodents but remain controversial in other species (Albina, 1995, Jungi *et al*, 1996; Vitek *et al*, 2006; Gross *et al*, 2014).

Activated microglial cells were thought to induce iNOS expression *in vitro* and *in vivo* in rodents (Dringen, 2005; Saha & Pahan, 2006). Activated iNOS was suggested to release a significant amount of NO that leads to neurodegeneration in diseases such as multiple sclerosis, AD and lysosomal disorders (Pannu & Singh, 2006).

**1.6.4.3 Haem oxygenase 1 (HO-1)**

Haem oxygenases are primarily responsible for degradation of Haem into equimolar amounts of labile iron, carbon monoxide and biliverdin (BV) (Tenhunen *et al*, 1968). Under physiological conditions, haem serves as a stable prosthetic group bound to Haemproteins. The free haem released from these proteins during oxidative stress has been proposed to cause further oxidative damage (Gozzelino *et al*, 2010). Two isozymes have been evolutionarily conserved (Tenhunen *et al*, 1968). HO-1 is the inducible form of haem
oxygenase and is thought to be regulated by oxidative stress (Kutty et al, 1994a; Choi & Alam, 1996). Cells that lack HO-1 expression show more susceptibility to oxidative stress (Poss et al, 1997; Yet et al, 2003; Bishop et al, 2004).

The role of HO-1 in oxidative stress is not explicit. Free haem was thought to cause oxidation through the Fenton reaction, in which the ferrous ion (Fe$^{2+}$) in free haem could be oxidised by H$_2$O$_2$ to form Fe$^{3+}$, a •OH and a hydroxide ion (Fenton, 1894; Poss et al, 1997; Yet et al, 2003; Bishop et al, 2004). Paradoxically, the resultant catalysis of free haem by HO-1 could also lead to oxidation. The accumulation of the free iron that is thought to underly the iron deposition theory of aging, AD, and PD, was also thought to contribute to the Fenton reaction (Schipper et al, 2009a). The ferritin and ferroportin iron sequestration pathways may obviate potential toxicity related to the intracellular accumulation of Haem-derived iron (Ryter et al, 2006; Zhang et al, 2014).

HO-1 is also associated with anti-inflammation, but the mechanism is also not clear. The anti-inflammatory actions of HO-1 have been related to regulation of ectopic expression of NF-κB-dependent cytoprotective genes and inhibition of the apoptotic MAPK signalling pathway (Gozzelino et al, 2010). Anti-inflammatory cytokines such as IL-10 and TGF-β could stimulate expression of HO-1, whereas pro-inflammatory cytokines such as INF-γ repress expression of HO-1, suggesting HO-1 may be implicated in the anti-inflammatory actions elicited by anti-inflammatory cytokines (Kutty et al, 1994b; Ryter et al, 2006).

The bile pigment (a soluble greenish pigment) produced by HOs (HO-1 and -2) catalysis, biliverdin (BV), can be converted by biliverdin reductase (BVR) to produce bilirubin (BR), a hydrophobic yellowish pigment that partitions to the lipid phase. BR is then excreted from cells and tissues and enters the blood by binding to albumin. It is removed from the serum exclusively by the liver, where it is metabolised to mono- and diglucuronides by hepatic glucuronyltransferases to become soluble and eventually passes from the liver through the bile to the faeces. In faeces, it is further degraded to urobilinogens by the reductive processes of intestinal microorganisms. The albumin-bound BR is thought to be an antioxidant through its ability of scavenging peroxyl radicals in plasma and the extravascular space (Stocker et al, 1987). The other by-product, carbon monoxide, is suggested to act similarly to NO by modulating guanylate cyclase to increase intracellular concentrations of the second messenger (cGMP) (Verma et al, 1993; Fukuda et al, 1996).
1.6.5 Neuroinflammation in neurodegeneration

The central nervous system (CNS) is considered to be immune-privileged, since the BBB and cerebrospinal fluid (CSF) form a barrier which restricts the entry of plasma proteins and immune cells to the brain. There is a lack of dendritic cells and lymphatic vessels in the brain (Perry, 1998). These features, along with perivascular macrophages, neuroglia and non-neuronal cells (eg. pericytes), are responsible for structural support, maintenance of homeostasis, and immune surveillance of the CNS. Following damage, activation of microglia and astrocytes can cause breaches of the BBB and consequently an influx of peripheral macrophages and lymphocytes which exacerbate the inflammatory response.

1.6.5.1 Glial activation

Astrocytes form a symbiotic connection with neurons in conjunction with oligodendrocytes in the brain parenchyma and affect neuronal functions by releasing neurotrophic factors, guiding neuronal development, contributing to neuronal metabolism and regulating homeostasis. Astrocytic foot processes are in close apposition to the abluminal surface of the microvascular endothelium of the BBB thereby helping to support its structural and functional integrity (Dong & Benveniste, 2001). They typically exhibit a star-shaped morphology, with many processes extending from their soma. A number of functions have been attributed to astrocytes, including the biochemical support of neurons and other cell types, involvement in the repair of the brain (scar formation) following mechanical or inflammatory injury and support of endothelial cells forming the BBB. Astrocytes, identified immunohistologically by the expression of the intermediate filament glial fibrillary acidic protein (GFAP), undergo proliferation and morphological change upon activation. Ablation of astrocytes in a forebrain stab injury in transgenic mice led to neurodegeneration, increased infiltration of blood-born inflammatory cells and phagocytic macrophages, and increased unguided outgrowth of nerve fibres (Bush et al, 1999). This study demonstrated clearly that astrocytosis is essential to maintaining the integrity of the BBB, and supportive of neuronal survival and neurite out-growth after CNS injury. However, astrocytosis has also been proposed to have a detrimental effect when chronically activated. Prolonged astrocytosis leads to aberrant expression of cytokines and chemokines that may be harmful to the neurons in neurodegenerative diseases (Dong & Benveniste, 2001).

Microglia are resident brain macrophages, derived from myeloid cells which migrated to the CNS during embryogenesis (Kettenmann et al, 2011). They are regarded as sensors of
pathology and become activated when there is a change of the environment such as the presence of infectious agents and changes of neuronal activity (Perry et al, 2010; Kettenmann et al, 2011). Microglial activation is a common hallmark of neurodegeneration (Perry et al, 2010). Microglia are normally in a resting state and become activated upon CNS insult and migrate to the sites of the insult by following chemotactic gradients. Upon activation, microglia release inflammatory mediators aiming for the protection and restoration of CNS homeostasis. The activated microglia could be phagocytotic and able to clear tissue debris, damaged cells or invaded bacteria/virus (Kettenmann et al, 2011). Early studies have suggested microglial activation facilitated neuronal regeneration, delayed neuronal death and supported neurogenesis (Streit, 2002; Ziv et al, 2006). In contrast, microglial activation has also been proposed to contribute to the progression of neuroinflammation and neurodegeneration. Neurotoxic effects of microglial activation following acute insults have been reported (Krady et al, 2005; Purisai et al, 2007). Upon activation, microglia may release neurotoxic molecules such as pro-inflammatory cytokines and ROS that are involved in the neurodegenerative process (Gao & Hong, 2008; Perry et al, 2010).

The opposing roles of microglial activation have been attributed to the plasticity and the distribution of microglia. Microglia are capable of switching between phenotypes, which are associated with either regulatory or detrimental functions, activated by different molecular cues (Perry et al, 2010). Depending on the types of microglial phenotypes, progression of the disease and the interplay with other cells in the CNS, functions of microglial activation could be quite opposite, either protective or threatening (Perry et al, 2010; Cherry et al, 2014). Moreover, regional differences in microglial populations, their inflammatory responses and their interactions with other cells, such as astrocytes, may influence the final outcome of their activation (Gao & Hong, 2008). For instance, microglia isolated from the subventricular zone (SVZ) or the cerebellum of adult mice have different influences on the rate of neurogenesis (Walton et al, 2006).

1.6.5.2 Neuroinflammation leads to the BBB breach

The BBB provides a physiological barrier between the blood and the CNS to prevent blood-borne pathogens and toxins entering the CNS. The BBB is located at cerebral blood vessels and the choroid plexus epithelium and is absent in the circumventricular organs (CVOs) (Weiss et al, 2008). CVOs are an integral part of neuroendocrine function in brain. They have
a rich vascular plexus with a specialised arrangement of blood vessels which lack BBB and allow diffusion of large molecules (Abbott et al., 2006; Weiss et al., 2008).

The BBB barrier is formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular end-feet. It acts as a physical barrier as a result of the tight junction proteins between the endothelial cells adjacent to the cerebral blood vessels, and between the epithelial cells of the choroid plexus (Weiss et al., 2008; Abbott et al., 2006). These restrict most para-cellular molecular traffic. Some lipophilic molecules can diffuse through the lipid membrane and some molecules take a transcellular route across the BBB. Nutrients such as amino acids and glucose are actively transported by a variety of transporters on the endothelial membranes while some transporters are energy-dependent and act as efflux transporters. Other transport pathways include receptor-mediated transcytosis and adsorptive-mediated transcytosis (Abbott et al., 2006). In addition, neurovascular units comprised of microvascular endothelia, astrocytes, pericytes, neurons, and the basal lamina form a functional unit which regulates cerebral blood flow through homeostatic mechanisms and thus maintains the BBB integrity (Abbott et al., 2006; Muoio et al., 2014).

It has been proposed that BBB disruption is an early consequence of neuroinflammation and multiple mechanisms have been hypothesised (González et al., 2014). The selectivity and permeability of the BBB is susceptible to damage by pro-inflammatory cytokines such as IL-1β and TNF-α (Stanimirovic et al., 1997; Matsumoto et al., 2014). Glial activation leads to the release of pro-inflammatory cytokines which trigger down-regulation of tight junction proteins in the endothelial cells of the BBB and result in increased permeability of the BBB (Nagyozi et al., 2010). Pro-inflammatory cytokines also up-regulate the expression of cell adhesion molecules (CAMs) on the endothelial cell surface. These interact with lymphocytic surface proteins such as lymphocyte function associated antigen 1 (LFA-1) on leukocytes and result in trans-migration of leukocytes into the brain (Weiss et al., 2008; González et al., 2014). A subpopulation of monocytes (a type of leukocyte) were reported to enter the brain and transform into microglia after BBB damage (Mildner et al., 2007). It has been suggested that pericytes are sensitive to TNF-α (Matsumoto et al., 2014). These cells seemed to be stimulated by TNF-α to produce a unique profile of cytokines and chemokines, which have been suggested to signal recruitment of immune cells to enter the BBB, as well as promoting microglial activation and proliferation (Matsumoto et al., 2014). Another in vitro study supported the notion that pericytes, under the influence of pro-inflammatory cytokines,
could produce more pro-inflammatory cytokines that facilitate neutrophil trans-migration (Pieper et al., 2013). Activated pericytes in vitro show phagocytic ability and could express pro-inflammatory proteins (eg. iNOS) and produce ROS and RNS (Pieper et al., 2014). Matrix metalloproteinases (MMPs) were thought to be elevated during inflammation to cause degradation of tight junction proteins and result in BBB breaches (Lakhan et al., 2013).

1.6.5.3 Other signalling molecules proposed to be involved in neuroinflammation

The PI3K signalling pathway plays a fundamental role in signal transduction in mammalian cells (Engelman et al., 2006; Vanhaesebroeck et al., 2010). It mediates adaptive immunity, by regulating the development, activation and differentiation of both B- and T-cells and activation of autophagy, as well as mediating the innate immunity (Beer-Hammer et al., 2010; Wang et al., 2011; So & Fruman, 2012). PI3K signalling is also involved in vesicle trafficking in cells and synaptic plasticity in mature neurons (Falasca & Maffucci, 2009; Knafo & Esteban, 2012). An alteration in PI3K signalling has been implicated in neurodegenerative diseases such as AD (O’Neill et al., 2012). Although the common PI3K signalling mechanism is known, the exact mechanism of PI3K signalling involved in neurodegeneration is not fully understood.

Recent studies suggest that progranulin and metallothioneins could be implicated in NCL disease mechanisms in pathways such as neuroinflammation and metal dysregulation respectively (Smith et al., 2012; Kanninen et al., 2013; Petkau & Leavitt, 2014). However, it is not clear what roles these molecules play in the proposed mechanisms.

1.6.6 The regulation of pro- and anti-inflammatory cytokines

Neuroinflammation is one of the key features in the pathogenesis of neurodegenerative diseases. In NCLs, progressive activation of astroglia and microglia precedes neuronal loss suggesting a causative link, but whether glial activation triggers neurodegeneration or is a stress response remains unclear (Oswald et al., 2005). Bi-directional communication between glia and neurons via pleitropic and multifunctional cytokines may influence the fate of neurons. A previous study showed a generalised and chronic up-regulation in anti- and pro-inflammatory cytokine gene expressions in the CLN6 affected cortex, concurrent with the initiation of neurodegeneration throughout the disease progression, suggesting an uncontrolled inflammatory response (Barry, 2011). This indicates a counter-active
mechanism in which anti-inflammatory cytokines fail to suppress inflammation thereby implying a rather complex and intricate signal transduction network in the disease.

Progressive and chronic elevation of anti- and pro-inflammatory cytokines in the ovine NCL brain links to the co-existence of trophic and toxic pathways (Barry, 2011). The common molecular mechanisms regulating these pathways include the transcriptional pathways of pro-inflammatory cytokines (NF-κB, JAK/STAT, and MAPK) as well as the inhibition of the pro-inflammatory pathway via inhibiting the transcription of pro-inflammatory cytokines (eg. SOCS3).

1.6.6.1 NF-κB

NF-κB (nuclear factor of kappa light polypeptide gene enhancer) is a eukaryotic transcription factor which was discovered in B lymphocytes in 1986 and named after its function in transcription of immunoglobulin kappa light chain (Sen & Baltimore, 1986). It was later found to activate expression of immune-related proteins such as cytokines and acute phase response proteins (Ghosh et al, 1998). The NF-κB family includes members of p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) in the presence of homo- or heterodimers, including p50-p50, p65-p65, p50-p65, c-Rel-c-Rel, p50-p50-IκB (Ghosh et al, 1998). The family members contain a distinct feature, a Rel homology domain (RHD) which is responsible for dimerisation, interaction with inhibitors and binding of DNA (Fig 1.3). The RHD also contains a nuclear localisation signal (NLS) for import into the cell nucleus. RelB, c-Rel and p65 contain a trans-activation domain (TD) therefore they have intrinsic transactivating potential which p50 and p52 homodimers do not have. Therefore, p50 and p52 have to form heterodimers with RelB, c-Rel or p65 to be transcriptionally active (Ghosh et al, 1998). Homodimers of p50 and p52 also appear to be repressive of gene transcription because they do not have TD domains (Ghosh et al, 1998; Zhong et al, 2002). The most common heterodimer of NF-κB found in cells is p65/p50, commonly referred as NF-κB (Ghosh et al, 1998).
Figure 1.3  **Protein domains of the NF-κB family.** The black bar indicates the Rel homology domain (RHD), TD represents the transactivation domain, GRR is the glycine-rich region and LZ the leucine zipper. The black arrows indicate where endoproteolytic sites on p100 and p105 which are processed into p52 and p50 (Ghosh *et al*., 1998).

NF-κB occurs in cells in a latent, inactive form which is bound by the inhibitor, IκB. Activation of NF-κB dimers is regulated by a canonical or a non-canonical pathway for members of the NF-κB family (Hayden & Ghosh, 2008). In the canonical pathway, a signal transduction pathway of inflammatory molecules such as TNF-α leads to phosphorylation of IκB kinase β (IKK β). The phosphorylated IκB dissociates from the NF-κB - IκB complex and is targeted for ubiquitination and degradation. Meanwhile, the NF-κB homo- or hetero-dimers translocates to the nucleus where they up-regulate specific genes containing a consensus sequence, 5'-GGGRNNYYYCC-3' (R: purine, Y: pyrimidine, N: flexible) (Ghosh *et al*., 1995; 1998; Müller *et al*., 1995; Hayden & Ghosh, 2008).

1.6.6.2  **JAKs**

Janus kinases (JAKs) are non-receptor tyrosine kinases and four family members have been found in mammals, JAK1, JAK2, JAK3 and TYK2. JAK1, 2 and TYK2 are ubiquitously expressed in cells whereas JAK3 was found to be expressed mainly in cells of the haematopoietic system (Ihle & Kerr, 1995; Yeh & Pellegrini, 1999; Heinrich *et al*., 2003). JAK kinases play a role in a variety of biological processes relating to haematopoiesis and the immune response (Haan *et al*., 2002). They are known to regulate cytokine signal transduction together with activation of transcription factors (STATs).
JAKs are approximately 1,100 amino acids long with molecular mass between 120kDa and 140kDa. The generic structure of a JAK kinase consists of 7 JAK homology (JH) domains ranging from JH1 at the carboxyl terminus to JH7 at the amino terminus (Wilks et al, 1991; Fig 1.4). These domains are functionally defined as FERM domains (JH7 and JH6), SH2-like domains (JH5-JH3), a pseudokinase domain and a kinase domain. The amino terminal of the FERM domain binds to the membrane –proximal box1/2 region of cytokine receptors (Zhao et al, 1995; Chen et al, 1997; Kohlhuber et al, 1997; Richter et al, 1998; Cacalano et al, 1999). The remaining domain of FERM was thought to facilitate and stabilise JAKs binding to cytokine receptors (Haan et al, 2008). The Src-homology 2 (SH2) like domain (JH3-JH5) was postulated to provide structural support for the FERM domain (Radtke et al, 2005).

The kinase domain mediates kinase activity via phosphorylation of tandem tyrosine residues in the activation loop. For instance, key tyrosine residues are Tyr1007 and Tyr1008 in JAK2; and Tyr 980 and Tyr 981 in Jak3 (Feng et al, 1997; Zhou et al, 1997). Phosphorylated tyrosine residues lead to enhanced catalytic activation and the expulsion of the activation loop from the active site to expose a docking site for inhibitors, such as suppressor of cytokine signalling protein 1 (SOCS1) (Rane & Reddy, 2000; Flowers et al, 2004; Lucet et al, 2006).

The pseudokinase domain retains considerable homology to the tyrosine kinase domain but lacks the functional motifs of typical kinase activity (Haan et al, 2012). The high conservation of the dual domain structure from the fly to mammals suggests functional necessity (Haan et al, 2012; sequence BLAST). Deletion or mutations of the pseudokinase domain led to defective JAK activation and downstream signalling (Russell et al, 1995; Candotti et al, 1997; Saharinen et al, 2000). The exact mechanism of how pseudokinase regulates kinase activity is unknown but one possibility is that the pseudokinase domain inhibits the kinase domain by an intramolecular interaction. A conformational change upon ligand-binding dimerisation of receptors and JAK kinases disrupts this interaction (Haan et al, 2012).
1.6.6.3 **STATs**

The signal transducers and activators of transcription (STATs) are well characterised JAK substrates. There are 7 genes in mammals responsible for encoding proteins STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 that are between 750 and 850 amino acids long (Kisseleva *et al.*, 2002).

The general structure of a STAT protein consists of an amino terminal domain (NH2), a coiled coiled domain (CCD), the DNA binding domain (DBD), a linker domain, an SH2/tyrosine activation domain and a carboxy-terminal transcriptional activation domain (TAD) (Fig 1.6). It has been suggested that the TAD domain exhibits divergence which contributes to the STAT specificity (Kisseleva *et al.*, 2002; Goenka *et al.*, 2003). Other features, including post-translational configuration, formation of dimers, and tissue-specific distribution, account for STATs specificity (Kisseleva *et al.*, 2002; Delgoffe & Vignali, 2013).

**Figure 1.5** **Representative features of STATs proteins.** Domains depicted: an amino-terminal domain (NH2), a coiled-coil domain (CCD), the DNA binding domain (DBD), a linker domain, an SH2 domain, and a transactivation domain (TAD). Post-translational modifications are indicated. *Grey balloon:* arginine methylation for modulation of interactions with epigenetic machinery. *Orange balloon:* a phosphotyrosine site for dimerization. *Black balloon:* a serine phosphorylation site for modulating gene target specificity and transcriptional activity. *Blue bubble:* a modification site for PIAS. Protein inhibitor of activated STATs proteins (PIAS) inhibits the activation and nuclear accumulation of STAT proteins (Delgoffe & Vignali, 2013).

The amino terminal domain (NH2) has been shown to mediate STAT dimer binding by stabilising dimer formation (Xu *et al.*, 1996; Vinkemeier *et al.*, 1996). A crystalised structure of
a tyrosine phosphorylated STAT1 dimer bound to DNA by Chen et al (1998), revealed a model of the binding mechanism of a STAT dimer to DNA: A core DNA binding element consists of two monomers from each STAT and each monomer consists of the SH2 domain, the linker domain, the coiled-coil domain and the DNA binding domain. This core DNA binding element clamps a traversing DNA fragment (Fig 1.6).

![Figure 1.6](image)

**Figure 1.6** A crystal structure of the core human STAT1 and DNA complex (retrieved from Chen et al, 1998).

The SH2 domain is highly conserved among the STATs and plays an essential role in STATs signalling. The domain contains a strictly conserved arginine residue, such as Arg-602 in STAT1, which recognises and docks to the phosphotyrosine on the receptor (Chen et al, 1998).

The surface of the coiled coil domain is mainly hydrophilic which enables interactions with other proteins such as co-activator proteins (Bhattacharya et al, 1996; Horvath et al, 1996; Zhang et al, 1999; Zhu et al, 1999). A small cluster of hydrophobic side chains associates with the binding of DNA (Chen et al, 1998).

The DNA binding domain recognises a GAS (gamma-activated sequence) element, which is an 8 to 10 base pair inverted repeat DNA sequence of 5'-TTC(N_{2-n})GAA-3' (N, refers to the number of nucleotides in between the palindromic sequence, Ehret et al, 2001). Other binding sequences of STAT dimers similar to a GAS element include ISRE (interferon-
stimulated response element) and GAS-like element or cis-inducible element (Aaronson & Horvath, 2002).

### 1.6.6.4 JAK/STAT

JAK-STATs comprise a signalling cascade involved in cytokine signal transduction. Four members of the JAK family (JAK1, 2, 3 and TYK2) and 7 members of the STAT family (STATS1, 2, 3, 4, 5A, 5B and 6) have been discovered (Campbell, 2005). The suggested mechanisms of binding and activation of the JAKs in the receptor complex are largely theoretical (Haan et al, 2012; Babon et al, 2014). Cytokines binding to their corresponding receptors trigger dimerisation of these receptors and recruitment of JAKs to juxtapose the cytosolic part of the receptors, in which JAKs phosphorylate STATs (Haan et al, 2012).

Constitutive expression of JAKs and STATs in the CNS is lower than in other tissues (Campbell, 2005; Nicolas et al, 2013). JAKs and STATs (especially JAK1, 2 and STAT1, 3, 6) seem to be expressed actively during brain development and decline when the brain reaches maturation (De-Fraja et al, 1998). Studies also show a correlation between the JAK/STAT pathway and those of neurogenesis and astrogliogenesis, probably mediated by various cytokines and growth factors (Bauer, 2009; Gómez-Nicola et al, 2011). Expression of STATs appears to vary in cell types: STAT1 is highly expressed in neurons and moderately in glial cells while STAT4 is thought to be restricted to infiltrated T-lymphocytes and macrophages (Maier et al, 2002). Under pathological influences, the expression of STATs is dependent on the stimulation of different cytokines and cell types.

### 1.6.6.5 MAPK

Mitogen-activated protein kinases (MAPKs) are one of the central signalling hubs activated in response to changes in the extracellular milieu. The well characterised MAPKs are the extracellular signal-regulated kinases (ERK1/2), C-Jun NH2-terminal kinases (JNK-1/2/3), p38 and ERK5 (Huang et al, 2009). These enzymes contain a conserved motif, TXY (T, threonine; Y, tyrosine; X, = glutamate in ERK, proline in JNK, or glycine in p38) in the activation loop of the kinases (Cuenda & Rousseau, 2007; Huang et al, 2009). MAPKs are fully activated by the dual phosphorylation of threonine and tyrosine in the conserved motif (Huang et al, 2009). MAPK kinase kinases (MKKKs) can be activated by physical and chemical stimuli to phosphorylate MAPK kinases (M KKs), which in turn phosphorylate MAP kinases (Cuenda & Rousseau, 2007; Huang et al, 2009). Activated MAPKs regulate key cellular events in the
cytoplasm by phosphorylation of membrane-associated and cytoplasmic proteins, including other kinases and cytoskeletal elements, as well as translocating to the nucleus to phosphorylate transcription factors for downstream gene expression (ter Haar et al, 2007; Huang et al, 2009).

The p38 MAPK pathway is suggested to be activated by stress to regulate an immune response, cell survival and differentiation (Cuadrado & Nebreda, 2010). The first p38α MAPK was discovered as a 38kDa protein phosphorylated on tyrosine when cells were exposed to inflammatory stimuli and stress (Han et al, 1994; Rouse et al, 1994). Specific inhibition of the p38MAPK pathway led to down-regulation of pro-inflammatory cytokines (Hill et al, 2008). Subsequently, three isoforms were discovered to be expressed in a tissue-specific manner. They are p38β in brain, p38γ in skeletal muscle, and p38δ in endocrine glands. P38α is ubiquitously expressed at a high level in most cell types (Cuadrado & Nebreda, 2010). The p38s exhibit some functional redundancy (Sorrentino et al, 2008; Yamashita et al, 2008).

The 3D structure of p38α MAPK contains a N- and C-terminal domain, an ATP binding site, a catalytic loop, a docking groove, and a substrate binding groove (ter Haar et al 2007). The docking groove is important for the binding of activators and substrates (Chang et al, 2002). Phosphorylation of the activation residues of p38α induces a conformational change and opens the substrate binding groove, in which the phosphorylation of the substrate takes place (ter Haar et al, 2007).

The p38 MAPK pathway was thought to promote pro-inflammation. An anti-inflammatory cytokine, TGF-β, seems to associate with cell apoptosis through activation of the TRAF6-TAK1 pathway (Sorrentino et al, 2008; Yamashita et al, 2008). Upon TGF-β binding to its corresponding receptor, TRAF6 (TNF receptor associated factor 6) was recruited to the ligand–receptor complex and led to auto-ubiquitylation of TRAF6. The autoubiquitylated TRAF6 promoted modification of TAK1 (TGF-β associated kinase 1), a member of the mitogen-activated protein kinase kinase kinase family, resulting in its activation. TAK1 then activates the p38MAPK pathway (Sorrentino et al, 2008). Pro-inflammatory cytokines such as TNF-α and IL-1β have also been suggested to activate p38MAPK via activation of the TRAF6-TAK1 pathway (Wang et al, 2001; Shim et al, 2005). The activated p38 MAPK up-regulates cytokine production by directly phosphorylating their associated transcription factors such
as ATF2 and CREB. Also, the pathway directly or indirectly stabilises and increases the translation of pro-inflammatory cytokines (Schieven, 2005).

1.6.6.6 **SOCS**

Suppressor of cytokine signalling (SOCS) proteins are intracellular molecules that negatively regulate pro-inflammatory cytokine signalling via inhibition of JAK/STAT. They play roles in innate and adaptive immunity by regulation of macrophage and dendritic-cell activation, T-cell development and differentiation (Yoshimura et al., 2007). SOCS proteins are generally present in low concentrations and expression increases rapidly upon cytokine activation (Alexander & Hilton, 2004). There are seven SOCS proteins (SOCS1-7). The inhibitory actions of SOCS proteins are facilitated by their structural features. The common features include a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxy-terminal 40-amino-acid peptide known as the SOCS box. The SOCS box is conserved in the SOCS family and interacts with components of proteosomal pathway so that the transducers bound by SOCS proteins can be targeted for degradation (Hilton et al., 1998; Zhang et al., 1999; Yoshimura et al., 2007; Babon et al., 2008). SOCS1 and 3 can directly inhibit JAK tyrosine kinase via their kinase inhibitory region (KIR) near the amino-terminus as well as preventing signalling complex assembly (Yoshimura et al., 1995; Matsumoto et al., 1997; Flowers et al., 2004; Waiboci et al., 2007; Dimitriou et al., 2008).

1.7 **Neurotrophic factors and the endosomal-lysosomal pathway**

Studies of chimeric animals produced by fusing normal and affected 16-32 cell embryos showed that there is intercellular correction in the CLN6 sheep despite the intracellular membrane location of the CLN6 protein. Chimeras showed normal-like or recovering physical growth and lack of cortical neurodegeneration despite up to 75% of the cells in the brain being genotypically affected (Barry 2011). Glial activation was reduced or absent in these animals but neurogenesis was unusually active throughout the cortical grey and white matter. Therefore, given the correct environmental milieu disease-affected and newly generated cells are amenable to correction by normal cells in CLN6 NCL, resulting in an amelioration of disease pathology.

It is reported that neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) are involved in the enhancement of adult neurogenesis,
either by direct effects on neuronal generation, or indirectly via the promotion of newly generated neuron survival (Palmer et al, 1995; Aberg et al, 2000; Lee et al, 2002; Jin et al, 2002).

Neurotrophic factors promote neuronal survival, stimulate axonal growth and play a key role in construction of the normal synaptic network during development (Yuen et al, 1996; Grimes et al, 1996). They help to maintain neural functions in adults, therefore any alterations in their local synthesis, transport or signalling could adversely affect neuronal survival and lead to neuronal death (Connor & Dragunow, 1998). Neurotrophins bind to their cell surface receptors and can be internalised and retrogradely transported in neurites (Miller & Kaplan, 2001; Bronfman et al, 2007). Neurotrophic factors binding to their corresponding receptors cause clathrin-coated vesicles to pinch from the membrane and move on to become “early endosomes”. From here, internalized receptors and ligands can be sorted to recycling endosomes and transported back to the plasma membrane for the re-use of receptors and re-release (and potentially re-use) of ligands. Alternatively, the early endosomes mature as late endosomes and eventually fuse with lysosomes for degradation (Bronfman et al, 2007). Alterations in the endosomal-lysosomal pathway could result in altered processing and degradation of these factors.

1.8 Research rationale

Glial activation occurs prior to neurodegeneration, which follows in a regionally specific manner, suggesting that neuroinflammation plays a central role in the pathogenesis of ovine CLN6 (Oswald et al 2005; Kay et al, 2006). Inhibition of neuroinflammation by minocycline was not successful (Kay & Palmer, 2013). This validates an investigation into underlying mechanism of the neuroinflammatory pathway in ovine CLN6. The study of the molecular mechanisms underlying glial activation revealed the unbalanced signalling of pro- and anti-inflammatory cytokines prior to neurodegeneration (Barry, 2011). However, it is not understood what could cause the abnormal inflammatory response. Therefore, the aim of this research is to further define the cytokine signalling pathways and if possible pinpoint the lesions in the neuroinflammatory pathway mediated by the uncontrolled cytokine response. If the lesions are found, more targeted drugs could be selected for therapeutic intervention of this devastating disease, and perhaps used in conjunction with gene therapies being developed for ovine CLN5 and CLN6.
A schema of the possible neuroinflammatory pathway summarised the selected signal transductions discussed previously and the elements potentially involved in neuroinflammation is shown in Figure 1.7 (Glass et al, 2010). The neuroinflammatory pathway comprises innate and adaptive immune responses. Briefly, the hypothesis is that the genetic defect caused by CLN6 mutations leads to glial activation, which produces pro- and anti-inflammatory cytokines via transcription regulation which in turn mediate the neuroinflammation (Raivich et al, 1999; Li & Verma, 2002; Kumar et al, 2003; Baker et al, 2009; Brown & Neher, 2010; Minogue et al, 2012). Pro-inflammatory cytokines may then play a role in maintenance of this glial activation. The pro-inflammation overwhelms neurons resulting in neuronal injury and cell death. Meanwhile, an anti-inflammatory pathway is activated, aiming to repair tissue damage and support neuron survival.

Activated glial cells may produce large amounts of reactive oxygen species and synthesise iNOS that produces nitric oxide, which forms peroxynitrite with superoxide that leads to oxidative stress thereby sustaining pro-oxidation and glial activation. Oxidative stress causes neuronal injury and cell death that also becomes a source of reactive oxygen species and causes more oxidative damage (Mander & Brown, 2005). An adaptive immune response is triggered by pro-inflammatory cytokines and leads to breaches in the BBB, allowing entry of the lymphocytic immune cells into the brain which could damage neurons when activated (Lim et al, 2007; Weiss et al, 2008; Saha et al, 2012; Groh et al, 2013). A loss of neurotrophic support for selective neuronal populations, such as reduced expression of neurotrophic factors and their receptors, may contribute to the pathology of neurodegenerative diseases (Henderson et al, 2000; Kopan et al, 2004; Blesch, 2006). Other possible signalling molecules, including PI3K, progranulin and metallothioneins will be investigated for their potential roles in the neuroinflammatory pathway (Kanninen et al, 2013; Götzl et al, 2014).
Figure 1.7  A schema of the neuroinflammatory pathway in ovine CLN6.

1.8.1 Research objectives

The overall aim is to identify points of causative lesions to aid in designing effective therapeutic options. This study will extend the understanding of the pathology in ovine CLN6 through the following objectives:

1. To characterise the cascade of activation of neuroinflammation and disease pathogenesis. Gene expression studies of selected oxidative modulators (MnSOD, iNOS and HO-1), transcription regulators of inflammation (JAK2, STAT 1 and 3, MAPK14, NF-κB, SOCS3) and neurotrophic and growth factors (BDNF, TrkB), as well as other potential inflammatory regulators (MT I/II/III and GRN) are proposed, based on quantitative (q) PCR using cDNA obtained from regions of affected and control brains of different ages. These will be complemented with immunohistochemical detection of the expression of target proteins on brain sections at different developmental stages of disease, and Western blotting of the protein expression of interest in selected regions.

2. To investigate PI3Ks expression by Western blotting analysis.

3. To investigate blood-brain barrier breaches by lymphocyte infiltration using immunohistochemical markers CD4 and CD8, and immunoglobulin deposition using IgG as a marker.
Chapter 2

General Materials and Methods

2.1 Animals

The sheep used in this study were part of a larger breeding program established to maintain a flock of NCL affected animals, and allow a longitudinal investigation into the underlying pathological mechanisms of NCL. The CLN6 model was first described 40 years ago after identification of the disease in two affected rams in a flock of South Hampshire sheep in the Manawatu (Jolly & West, 1976). The New Zealand South Hampshire CLN6 sheep flock is maintained by a programme of crossing homozygous affected rams with heterozygous carrier ewes, resulting in a 1:1 ratio of affected: heterozygous offspring (Kay et al, 1999). Outbreeding programmes over the years introduced heterozygous ewes attained from crosses between normal Friesian, Finn and Coopworth ewes and affected South Hampshire rams to improve the health and reproductive performance. The sheep are maintained under normal New Zealand pastoral conditions on university farms and animal procedures are carried out in accordance with the New Zealand Animal Welfare Act, 1999 and sanctioned by the Lincoln University Animal Ethics committee.

Since 2004, a discriminatory c.822G>A polymorphism in the CLN6 gene has been used to determine genotype. This is in the coding region of CLN6, 111bp downstream of the 5’ end of exon 7 (Tammen et al, 2006). It is not disease causing but all the homozygous CLN6 affected South Hampshire sheep only carry the A allele, which is a silent substitution to the third base of a triplet coding for alanine. The substitution blocks the cleavage site for the restriction enzyme Hae II and therefore causes the differential sizes of the PCR fragments on 4% agarose gel after enzyme digestion (Fig 2.1, Tammen et al, 2006). The South Hampshire flock is maintained such that all the normal alleles carry the G base and all the affected alleles are A. Hence, an A/G restriction enzyme test on DNA extracted from the blood of young offspring is useful for routine preclinical diagnosis. The disease causing mutation is at the other end of the reading frame, being a 402bp deletion and a 1bp insertion that includes the 83bp sequence of the complete first exon of ovine CLN6 (Section 1.5.2; Ismail, 2014). The affected genotype is corroborated by the development of disease symptoms or confirmation of storage body accumulation in tissues post mortem (Jolly et al, 1982, Oswald
et al 2005; Frugier et al, 2008). Clinically normal CLN6 heterozygous South Hampshire and CLN5 heterozygous Borderdale sheep are used as experimental controls. No sign of disease has ever been noted in heterozygous animals.

Figure 2.1 The CLN6 PCR product of 251bp was cleaved by restriction enzyme in South Hampshires. Lane 1. Molecular marker, a size standard with 300, 200 and 100 bp bands Lane 2. Control sheep (GG), yielding products of 117 and 67 bp after enzyme digestion. Lane 3 and 4. Carrier sheep (GA), three bands of 184, 117 and 67 bp are visible. Lane 5. Affected sheep (AA) shows two bands of 184 and 67 bp (Tammen et al, 2006).

The affected animals and the age matched controls were sampled at critical timepoints determined by marked pathological features in the disease progression noted previously, and summarised in the simplified diagram below (Fig 2.2; Section 1.5.2; Jolly et al, 1982; 1989, Mayhew et al, 1985, Oswald et al; 2005; Kay et al, 2006). Glial cells are activated as early as prenatally (Kay et al, 2006). Neurodegeneration, predicted by glial activation and reflected by changes in neuron populations, initiates at 6 months. Clinical signs are obvious by 9 months and are associated with visual impairment and behavioural changes. Advanced disease reflects a severe neurological phenotype, concomitant with the complete loss of vision, which is a result of atrophy of the occipital cortex and loss of photoreceptors in the retina by 18 months. The brain is severely atrophied and animals reach maximum life expectancy at 24 months.
Figure 2.2  Animal samples collected from timepoints during disease progression. The timepoints are highlighted by different colours.

The gene expression profiles of pro- and anti-inflammatory cytokines had been done on animals at 6, 9, 18 and 24 months (Barry, 2011). The present study supplemented these cytokine expression profiles with those of animals aged 2 months. Expressions of proposed neuroinflammatory genes were measured at all the critical timepoints. The same animals were used for all of the genes investigated.

2.2 Sample collection

2.2.1 Brain collection for immunohistochemistry and western blotting

An archival collection comprises sections from CLN6 affected and age-matched control samples at the ages of 2, 6, and 12 months, as well as at advanced disease (18 months and older). The fresh frozen brain bank contains slabs from the CLN6 ovine model at the ages of 2, 6, 9, 12 and 18+ months collected for regional RNA or protein studies.

Brains collected for immunohistochemistry were perfusion fixed via the carotid arteries with phosphate buffered saline (PBS, pH7.4, 37°C) then with 10% formalin in 0.9% NaCl. They were equilibrated in a solution of 30% sucrose, 10% ethylene glycol and 0.9% NaCl, and stored frozen at -80°C for subsequent tissue sectioning.

Sequential 50μm sagittal sections (from the left hemisphere) were obtained by the method of Oswald et al (2005), and coronal sections (from the right hemisphere) were cut and classified according to the sheep brain atlas from Michigan State University (https://www.msu.edu/~brains/brains/sheep/index.html). The cut sections were
photographed and stored frozen in cryoprotectant ready for immunohistochemical examination.

2.2.2 Brain collection for quantitative real-time PCR and Western blotting

Affected sheep aged 2, 6, 9, 18 and up to 24 months and age matched controls were sacrificed by exsanguination. Brains were removed from the skull and bisected along the midline. Half hemispheres were then cut sagittally into 6mm slabs and snapped frozen in liquid nitrogen. The slabs were assigned as 4 to 1 from the slab closest to the midline to the most lateral. Since the affected brains atrophied greatly at the advanced disease stage, the number of slabs gained was less. The slabs were stored at -80°C.

2.2.3 Archived tissue and RNA integrity

This study relied greatly on the real-time quantitative PCR (RT qPCR), the accuracy of which is correlated with the quality of the starting materials. The selected snap-frozen brain sections for this longitudinal study were from multiple timepoints and different time elapsed between the acquisitions of tissues of different ages and RNA extraction. In this scenario it is possible that the quality of RNA extracted from the archived tissues could vary as extended storage has a negative impact on the RNA integrity and thus affect the reliability of qPCR measurement. Other factors that could affect the RNA quality include delays of processing time and temperature variation during storage (Lee et al, 2012).

A stringent protocol was applied to tissue collection. Brains were removed and processed in liquid nitrogen immediately upon sacrifice at site. Labels and catalogues were prepared prior to the tissue collection to ensure that frozen tissues were readily catalogued and stored in assigned spaces to ensure rapid freezing and accurate storage and retrieval.

The available and acceptable techniques for checking the RNA integrity were used in daily practice in the lab. Purities of the extracted RNAs were measured by A260/A280, and A260/A230 as a secondary measure of purity. Analysis of RNA integrity by gel electrophoresis was also routinely used to determine the ratio between the quantities of the ribosomal RNA molecules (28S and 18S rRNA at a ratio of 2:1). Both these traditional methods are not entirely reliable. The A260/A280 does not reveal RNA degradation. The ratio of the ribosomal RNA fragments is not the best indicator of mRNA quality since the ribosomal fragments are independent of the transcripts that are to be measured. They do
however provide a good start and good lab practice should have avoided the induction of the RNA degradation from exogenous contaminants (Fleige & Pfaffl, 2006; Nolan & Bustin, 2008).

Using more than one reference gene as an internal control for normalisation of variability between samples and targeting short amplicons is essential to prevent complications arising from sub-optimal RNA quality (Fleige & Pfaffl, 2006). In the present study, expression of the reference genes (GAPDH and ATPase) showed robust amplification kinetics and stable expression between the samples and between qPCR runs. This indicated reliable RNA quality of all the samples used for this study. Meanwhile, gene expression profiles from partially degraded RNA samples has been shown to be highly similar to those of intact samples and RNA samples of sub-optimal quality (Schoor et al, 2003). It has been reported that using RT qPCR products between 20-250bp could avoid concerns of RNA quality (Fleige & Pfaffl, 2006). Fragmentation of RNA within the shorter product is a rare event unless the RNA is severely degraded and random priming during cDNA synthesis should be able to capture all the RNA fragments (Schoor et al, 2003).

The exogenous inhibitors from reagents for nucleic acid extraction or endogenous inhibitors from the biological samples such as bile salts, urea, haem, heparin or IgG could be problematic and cannot be corrected by internal reference genes (Fleige & Pfaffl, 2006; Nolan & Bustin, 2008). However, these problems are more likely to occur in RNA extracted from formalin fixed and paraffin embedded tissues than from fresh-frozen tissues (Nolan & Bustin, 2008).

2.3 Quantitative real-time PCR

2.3.1 RNA extraction and single-stranded cDNA synthesis

The methods of RNA extraction, single-stranded cDNA synthesis and quantitative PCR of the genes of interest in this study were adapted from Barry (2011).

The most medial slab of each brain was used for the RNA extractions. Tissue samples, 30mg, from the frontal, parietal and occipital cortices were collected using a sterilised scalpel. Total RNA was isolated from each sample using the Qiagen RNeasy Mini RNA extraction kit (Qiagen, Hilden, Germany). Traces of genomic DNA were removed by treatment with the RNase-Free DNase kit (Qiagen, Hilden, Germany). The concentration (μg/μl) and purity
(260/280nm) of RNA were measured by a spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). RNA concentrations were determined by the optical density (OD)$_{260}$ multiplied by the RNA factor (40):

$$40 \times \text{OD}_{260} \text{ of the sample} = \text{concentration of RNA (μg/ml)}$$

RNA integrity was checked by separating 4μl of total RNA on 1.5% agarose gels in TBE (Appendix A.4) with RedSafe (iNtrON Biotechnology, Korea) 40min, 80V. An image was captured using a GelDoc XR (Bio-Rad, USA) imaging system. Intact RNA was indicated by two crisp bands of ribosomal RNA species 28S and 18S (Fig 2.3).

![Image of RNA integrity](image)

**Figure 2.3** A representative image of the RNA integrity of a brain sample.

Single stranded cDNA was synthesised from 450ng of total RNA in two 20μl reactions using Superscript III reverse transcriptase (Invitrogen, USA) as per instructions. RNA was diluted to a total volume of 13μl with RNase free water, 1μl of 10mM dNTPs (Invitrogen) and 2μl of 50μM random hexamers (Invitrogen) and incubated, 65°C, 5min after which samples were submerged in ice for 1min. A first strand synthesis master mix consisting of 1X First Strand buffer, 200U Superscript III polymerase and 40U RNaseOut recombinant ribonuclease Inhibitor (Invitrogen) was prepared and a 7μl aliquot added to each sample. All PCR reactions were carried out on a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany). An incubation program was used: 1 cycle at 25°C for 5mins, then at 50°C for 50mins and at 70°C for 15mins. Following cDNA synthesis, replicate samples were pooled and 5μl aliquots frozen at -20°C. The remaining RNA samples were stored at -80°C.
2.3.2 Primer design

The mRNA sequences of target genes were obtained from data published on the National Centre for Biotechnology Information (NCBI) GenBank database. Both external and internal primers were designed using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The external primers were designed to produce a larger target fragment (approx. 500bp) for insertion into a plasmid. The internal primers (approx. 100-200bp) were designed to flank a smaller sequence within the larger target fragment for qPCR reactions.

The gene specific primers were synthesised and supplied lyophilized (Invitrogen). A stock concentration of 50μM of each primer was diluted in sterile TE (10mM Tris-HCL, pH 8.0, 1mM EDTA). A working solution was further diluted from the stock in sterile TE to 5μM. The stock and the working solutions were stored at -20°C.

Optimization of qPCR reactions was carried out to determine the optimal annealing temperature (Tm), cycle number and primer concentration prior to full scale qPCR experimentation, and for confirmation of primer specificity. The reaction was in a standard 20μl mixture containing 0.125μM of forward and reverse primers, 0.125mM dNTPs, 2μl 10X buffer, 2.5mM MgCl₂ and 1U Taq DNA polymerase (Qiagen). The PCR cycles used were 95°C for 10min, 35cycles of 95°C for 30s, 57°C for 30s and 72°C for 30s, followed by 72°C for 5min. Sharp and crisp bands were shown on 1.5% agarose gels (1.5% agarose gel in TBE with 1X RedSafe) at the expected molecular weights using 5μl of the 1Kb Plus DNA ladder (1μg/μl, Invitrogen) for reference. The products using external primers were sequenced and their sequences aligned with the predicted flanked mRNA sequences to confirm the specificity of primers.

2.3.3 Plasmid generation

For the generation of plasmids, the ovine cDNA fragments of target genes were inserted into a plasmid vector. Large amounts of successfully constructed cDNA were able to be grown and prepared in sufficient stock, which was later serially diluted for the standards. The same stock was used throughout the experiments to minimise interassay variations.

PCR products were separated on 1.5% agarose gels, excised and purified using the AxyPrep DNA gel extraction kit (Axygen Scientific Inc, CA, USA). The gel slices containing the DNA fragment of interest were excised under ultraviolet illumination and heated to 75°C in the
supplied buffer to melt the agarose. The solubilised DNA was washed and eluted in 30µl of eluent (2.5mM Tris-Cl, pH 8.5) on miniprep columns, supplied in the AxyPrep DNA gel extraction kit. Three µl of each purified DNA was run on 1.5% agarose gels for estimation of the concentrations and some was sent for sequencing (as indicated in 2.3.4), using the external primer pair to confirm the correct PCR product.

Insertions of 50ng of product into the sequencing vector pGEMT-easy (Promega Corporation, Madison, WI, USA) were completed by the incubation with ligation reaction mixture overnight at 4°C. Each 10µl ligation reaction mixture contained a 1:1 ratio of pGEMT-easy vector and insert, 2X rapid ligation buffer and 3U of T4 DNA ligase.

A microfuge tube containing 2µl of ligation mix and 50µl of competent cells (Escherichia coli DH5α, Invitrogen) were placed on ice for 20min, followed by heat shock at 42°C, 2min, and then immediately put on ice for a further 2 min. Super optimal broth medium (SOC), 950µl, was added and the transformation mix incubated in a shaking incubator at 850rpm, 90min, 37°C. Meanwhile, selective lysogeny broth (LB) agar plates were pre-warmed at 37°C for later usage. These selective LB agar plates contained of 100µg/ml ampicillin (Duchefa Biochemie B.V, Haarlam, Netherlands), 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) and 50mg/ml X-Gal (Quantum Scientific, Milton, Qld, Australia).

A 100µl and a 200µl transformation of competent cells with the ligated plasmid were plated onto separate LB agar plates and incubated at 37°C overnight. Following incubation, a single white colony from one of the LB + ampicillin plates was used to inoculate a 20µl PCR mix to check for the insertion of the intended sequence. A standard PCR was performed. Insertion was indicated by the appearance of a single band of the expected size. A 1ml starter culture (LB broth, 100µg/ml ampicillin) was inoculated with the selected colony and was grown for 4h, 37°C. This starter culture was then added to 10ml of LB broth containing 50µg/ml ampicillin and grown up overnight at 37°C in a shaking incubator at 250rpm. Several 500µl aliquots of the culture was mixed in glycerol and stored in cryotubes at -80 °C.

Isolation of plasmid DNA was completed by the AxyPrep plasmid miniprep kit (Axygen). A 1.5ml sample of the remaining culture was centrifuged to pellet cells, which were resuspended in the supplied buffer, lysed, and centrifuged for 10min, 12,000rpm, to pellet the bacterial DNA, protein, and cell debris. The resultant supernatant was transferred to a spin column, washed and centrifuged to adsorb plasmid DNA to the silica-gel membrane.
Plasmid DNA was eluted in 60μl eluent (2.5mM Tris-Cl, pH 8.5) and quantitated in a Nanodrop spectrophotometer at 260nm. The concentration of plasmid DNA was calculated from the optical density (OD)₂₆₀ multiplied by the DNA factor (50):

\[ 50 \times OD_{260} \text{ of the sample} = \text{concentration of DNA (μg/ml)} \]

The equation for the corresponding copy number of plasmids is as follows:

\[ (X \text{ g/μl DNA} / [\text{plasmid length in bp x 660 Daltons}]) \times 6.022 \times 10^{23} = Y \text{ molecules/μl} \]

Sequencing of the insert used the universal M13 forward (5’-GTA AAA CGA CGG CCA GT-3’) and reverse (5’-CAG GAA ACA GCT TAT GAC-3’) primers within the vector.

2.3.4 Sequencing reaction

A mix consisting of 5ng purified plasmids, 0.5μl BigDye of terminator premix (ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit, P/N 4336917, USA), 2μl half term buffer (BigDye Terminator v3.1 5x sequencing buffer, P/N 4336697) and 1μl (5pmol/μl) primer, was made up to 10μl with water. Sequencing was done at Lincoln University on an ABI Prism 3130xl Genetic Analyser with a 16 capillary 50cm array installed, using Performance Optimized Polymer 7 (POP7).

The post sequencing reaction clean-ups used the Agencourt CleanSEQ+ Sequencing Reaction Clean-up system (Agencourt CleanSEQ Dye-Terminator Removal from Beckman Coulter (P/N APN 000121, USA)). Agencourt CleanSEQ contains magnetic particles in an optimised binding buffer to capture sequencing extension products. Unincorporated dyes, nucleotides, salts and contaminants were removed using a simple washing procedure.

Alignment confirmed the correct insertions of the cloned sequences against target mRNA sequences via the GeneDoc multiple sequence alignment program (Nicholas and Deerfield, 1997). The remaining plasmid DNA was diluted to 10⁹ copies, aliquoted and stored at -20°C.

2.3.5 Selection of reference genes for quantitative PCR

Normalisation with suitable reference genes is important for reliable quantification of mRNA transcripts. The reference genes should be endogenously expressed in samples, acting as internal controls to eliminate errors arising from variation in the amount of starting materials, enzymatic efficiencies and tissue types (Bustin, 2000; Vandesompele et al, 2002).
The selection of the reference genes in this study was previously validated by Barry (2011). There are common reference genes but they are not universally used for all tissues. The gene expression can vary between experiments, tissue types and conditions of tissues (Schmid et al, 2003; Schmittgen & Zakrajsek, 2000; Radonić et al, 2004; Huggett et al, 2005; Robinson et al, 2007). GAPDH, RPLPO and ATPase were selected and validated for their reported stability in similar studies (Tammen et al, 2006; Houweling, 2009; Woodall et al, 1997). GADPH (glyceraldehyde 3-phosphate dehydrogenase) is an enzyme in glycolysis responsible for catalysing the conversion of glyceraldehyde 3-phosphate to 1, 3-bisphosphoglycerate and also implicated in transcription activation, apoptosis and axonal transport (Tarze et al, 2007). Genes for the large ribosomal protein P0 (RPLP0) and the ubiquitous ATPase have been used as reference genes in previous studies (Szameit et al, 2008; You et al, 2008; Houweling, 2009; Riley et al, 2008; Fakioglu et al, 2008; Woodall et al, 1997; Knight et al, 2007).

Expressions of candidate genes were analysed in the brain regions in the animals from different ages and genotypes. They were selected for their stability, determined by a measure of gene expression stability (M) in the Microsoft EXCEL application geNorm 3.5 (Vandesompele et al, 2002; Barry, 2011). The lower the value of M, the higher expression stability is (Vandesompele et al, 2002). GAPDH and ATPase were selected for their better expression stability in all the experimental animals. A normalisation factor (NF) was calculated in geNorm, then used as a multiplication factor to normalise copy numbers for the genes of interest (Vandesompele et al, 2002, Barry, 2011).

2.3.6 Quantitative real-time PCR

The standard curve method was used to determine the relative gene expressions of the inflammatory mediators (outlined previously) in the affected and control sheep at 2, 6, 9, 18 and 24 months of age, as has been done for TNF-α, IL-β, IL-10 and TGF-β in the CLN6 affected animals (Barry, 2011). Control (n=2) and age-matched affected (n=2) animals were used for each timepoint.

qPCRs were performed using the iQ SYBR Green Supermix (Bio-Rad) or a homemade mix, containing essential reagents (Invitrogen) including 10X buffer, MgCl2, dNTPs, and 10X SYBR green and appropriate internal primers. These qPCRs were performed using the iCycler iQ real-time PCR detection system (Bio-Rad) real-time machine. SYBR green (Invitrogen) was
supplied as a 10,000X concentrated stock then diluted to 10X working solutions in sterilised TE, pH 7.5. Both stock and working solutions were stored at -20°C. Serial 1:10 dilutions of plasmid containing the gene of interest at concentrations ranging from $10^0$ to $10^2$ copies per μl were run in parallel with each series of samples, allowing the generation of a standard curve and copy number per μl of sample to be calculated automatically by the software.

A general thermal cycling condition was set: 95°C for 15min, 40 cycles of 94°C for 30s, a range of temperature of 55°C-59°C for 30s and 72°C for 30s. Melting curve analyses were performed at the end of each PCR run to verify the specificity of the PCR products. A melt curve thermal condition consisted of 10s incubation at 60°C, followed by a 0.5°C increase in every subsequent cycle, to a maximum temperature of 99°C.

The amplification efficiency of the qPCR reaction for each gene was calculated from the standard curve using the equation, $E = 10^{-\frac{1}{slope}} - 1$ (Wong and Medrano, 2005). Minor differences in PCR efficiency between different runs were corrected for by using the same dilution series of the standard curve. The linearity of the relationship between the threshold values ($C_T$) and the logarithm of the DNA concentrations was monitored from the $R^2$ value. All standards and samples were run in triplicate in the same plate for each gene and each assay also included a non-template control to monitor any contaminations. The threshold values for each sample and baseline cycles were set automatically. The copy number per μl of sample was extrapolated by reading the $C_T$ value for that sample off its respective standard curve to obtain a log concentration value. The anti-log of that figure gave the relative concentration of the particular gene for that sample. This calculation was carried out automatically by the software.

Data were normalised to the geometric mean of reference genes GAPDH and ATPase. The same housekeeping genes were verified in a previous study (Barry, 2011).

### 2.3.7 Statistical analysis

Normalised gene copy numbers were imported into GenStat 15 (VSN International Ltd, Hempstead, UK), log transformed and analysed by the restricted maximum likelihood method (REML) and variance component analysis with Wald and F statistics to determine sources and sizes of variability caused by genotype, brain region, or age. Paired t-test analyses were also performed in Microsoft EXCEL to determine the variance between genotypes at each time point and the variance between time points in animals of the same
genotype. Probability values of $P < 0.05$ were considered statistically significant between genotypes and ages. Mean and standard error of the mean (SEM) data were back-transformed from the log data for visual representation on scatter plots using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). The statistical analytical methods were adapted from a previous study of cytokine expression (Barry, 2011) and performed on all gene expression data collected in this study.

2.4 Immunohistochemistry

Titrations of primary antibodies in phosphate-buffered saline containing 0.3% Triton X-100 (PBST) with 10% normal goat serum (NGS) were initially tested to determine an optimal concentration for staining proteins of interest, using sections likely to express the protein of interest in abundance. The target sections were triple rinsed in PBS to ensure dilution and subsequent removal of the cryopreservative. Endogenous peroxidise activity was quenched by incubation in 1% H$_2$O$_2$ in PBS for 20-30min prior to three further washes with PBS. Nonspecific protein binding was blocked in 15% NGS in PBST for 1h. Sections were then incubated in the chosen primary antibody dilution (in 10% NGS in PBST) for 8h at 4°C with gentle agitation on the rocker, followed by three washes with PBS. Two control brain sections (randomly chosen) from which primary antibody and secondary antibody were omitted respectively, were included in the process.

Sections were then incubated in biotinylated secondary antibody in 10% NGS in PBST at a 1:1000 dilution for 2h at RT on a rocker. After 3 washes in PBST, sections were incubated in ExtrAvidin-Peroxidase conjugate (Sigma, USA), diluted 1:1000 in PBST for 1.5h. Colour was developed by the addition of 0.2mg/ml 3, 3'-diaminobenzidine (DAB, Sigma, USA), diluted in PBS, containing 0.01% H$_2$O$_2$ for 7min. The reaction was stopped by adding a few drops of ice cold PBS. Sections were then washed with PBS three times and mounted on glass slides in chrome alum (5% gelatine, 0.05% chrome potassium sulphate, 0.05% sodium azide, all in deionised water). They were dried overnight on a slight angle, washed in 100% ethanol for 10 min and then transferred to xylene for 30 min. Slides were cover-slipped using DPX mountant (BDH Chemicals Ltd, Poole, England) and allow to dry overnight.
Table 2.1  Secondary antibodies used for immunohistochemistry analyses

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Conjugate</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit IgG (whole molecule)</td>
<td>Biotin conjugated</td>
<td>B7389, Sigma, USA</td>
</tr>
<tr>
<td>Anti-Mouse IgG (Fab specific)</td>
<td>Biotin conjugated</td>
<td>B7151, Sigma, USA</td>
</tr>
</tbody>
</table>

Digital images of stained sections were acquired with an Eclipse 50i microscope (Nikon, Tokyo, Japan) and a Nikon digital sight DS-U3 camera using NIS-Elements microscope imaging software (BR3.2, Nikon). The microscope lamp intensity, exposure time, condenser aperture setting, video camera setup and calibration, and the use of the neutral density filter were kept constant for capturing all images of a particular immunostain. Digital images were saved as .tiff files and figures and photomontages prepared in Corel Photopaint 12 (Corel Co., Ontario, Canada).

2.5 Western blotting

2.5.1 Protein extraction

Western blotting was also used to monitor the expression of selected proteins in brain homogenates from selected regions at different ages (further specified in the following chapters).

Grey matter was dissected from the frontal, parietal and occipital cortices and homogenised in extraction buffer (adjusted for different cellular fractions, shown in the following chapters) with a D130 homogeniser (LabServ, Ireland). The extraction buffer mainly contained phosphatase inhibitors (0.1M NaF, 1mM β- glycerol phosphate, 1mM Na3VO4) and protease inhibitor cocktails (Complete Mini, Cat# 11836153001, Roche Diagnostics, IN, USA). Homogenates were placed on ice for 20 mins then centrifuged in at 24,000g, 4°C, 15min. The supernatants were collected, aliquoted and stored at -80°C. The protein concentrations in the supernatants were measured by bicinchoninic acid (BCA) assays (Pierce Biotechnology, Rockford, IL, USA), described by Coligan et al, (1995). All samples were diluted 1:10, 1:50 and 1:100 with H2O and 10µl samples assayed in triplicate. Samples were incubated for 30 min, 37°C, with 200µl of BCA solution and compared to a standard curve generated using
dilutions of a 2mg/ml albumin standard to provide concentrations ranging from 0.0625mg/ml to 1mg/ml. Absorbance at 562nm was measured on a Fluostar plate reader (BMG Labtechnologies, Offenberg, Germany) and blank lysis buffer was analysed to provide a zero value.

2.5.2 Protein electrophoresis and transfer

Suitable amounts of total protein were mixed with 1X LDS loading buffer (0.2mM DTT, 2% LDS, bromophenol blue in Tris-HCl, pH 8.4) and separated by 12% LDS-polyacrylamide gel electrophoresis (PAGE) (acrylamide: bisacrylamide = 37.5:1). Protein was transferred to a Hybond C-extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) in a Bio-Rad Mini Trans-Blot electrophoresis transfer cell at 500mA, 115min, 4°C in an ice packed cooling unit containing ice-cold transfer buffer (25mM Tris, 192mM glycine).

2.5.3 Western blotting analysis

Membranes were rinsed in Tris buffered saline (TBS, 0.1M Tris-HCl, pH 8.0, 0.15M NaCl) after transfer, blocked in 5% blocking buffer (5% bovine albumin serum in TBST, 10mM Tris-HCl, 150mM NaCl, 0.1% Tween-20, pH 7.5) for 1h at RT, and rinsed in TBST. Primary antibody was initially tested and was diluted in 3% blocking buffer (3% bovine albumin serum in TBST). Membrane was incubated in primary antibodies at 4°C overnight. After three washes in TBST, the membranes were incubated in suitable secondary antibodies (Table 2.1), diluted 1:10,000 in 3% blocking buffer. The membrane was rinsed in TBST, 10min and in TBS, 10min. Subsequently, ExtrAvidin peroxidase (Sigma, USA) was diluted 1:10,000 in TBS, then applied to the membrane for 30 min, RT. Following four changes in TBS, antigens were detected by enhanced chemiluminescence using the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology). Exposure to BioMax MS Film (Eastman Kodak Company, Rochester, NY, USA) was carried out in an autoradiography cassette. The exposure time for each antigen was kept consistent for all samples to ensure that variation in band intensities did not arise from differing exposure times between blots. Films were developed with X-ray film developer and fixer solutions (Kodak, Japan) and subsequently scanned on a flatbed photo scanner (HPG3010, Hewlett-Packard, Palo Alto, CA, USA) at 600dpi and saved as JPEG files.
Chapter 3
Characterisation of key oxidative stress-responsive genes and gene products in ovine CLN6

3.1 Introduction

3.1.1 Lipid peroxidation

The storage bodies in Batten disease show lipofuscin-like fluorescence and have a similar ultrastructure to age pigment (ceroid), which led to the term neuronal ceroid-lipofuscinoses (Zeman & Dyken, 1969). However, the storage bodies occur not only in the nervous system but also in many visceral tissues. Therefore, “neuronal” is misleading and has been dropped from the name in some veterinary pathology contexts (Palmer, 2015). Because of the fluorescent properties of the storage bodies, the composition of the storage bodies had been proposed to be the products of lipid peroxidation. The idea was that lipid peroxidation of polyunsaturated acids generates malonaldehyde, which in turn reacts with amino groups of the proteins or phospholipids to form the fluorophors (Chio & Tappel, 1969 a, b). Lipid peroxidation had become a focus in the early studies.

Antioxidants including vitamin B2, vitamin B6, vitamin C, vitamin E, methionine, butylated hydroxytoluene, fish oil and sodium selenite were used either individually or in a cocktail to treat JNCL patients (Bennet et al, 1988; Santavuori et al, 1989; Westermarck et al, 1997), but did not ameliorate disease progression.

Compositional analyses of the isolated storage bodies showed that they had normal lipid profiles of lysosomes and a predominant and abnormal storage of small molecular weight protein later identified as subunit c of mitochondrial ATP synthase (Palmer et al, 1986a;b; 1988; 1989a; 2002; 2015). Storage bodies dissolved in 1% lithium dodecyl sulphate (LDS) did not fluoresce, indicating the absence of intrinsic fluorophors (Palmer et al, 1993; 2002). The observed fluorescence of the storage bodies is now postulated to be an aggregate property, possibly caused by the light defracting from the subunit c aggregates which were caused by the highly hydrophobic nature of subunit c (Palmer et al, 2002, Palmer, 2015). It was also noted that the storage bodies were enriched with transitional metals, which were in widely differing concentrations in the affected tissue, and this enrichment was only correlated with
the tissue specificity of the metal metabolism (e.g., the elevated copper concentration of storage bodies in liver) (Palmer, 1987). The presence of metals did not facilitate lipid peroxidation since there was no evidence of lipid peroxidation (Palmer, 1987; Palmer et al., 1988; Jolly et al., 2002).

The abnormal accumulation of the subunit c was proposed to arise from a mutant expressed pseudogene or the trimethylation on lysine 43 (Palmer et al., 1990; Medd et al., 1993). These ideas were discounted when the genes and their levels of expression were found to be normal in the affected sheep and the trimethylated lysine 43 a normal post-translational modification of all subunit c (Medd et al., 1993; Chen et al., 2004; Walpole et al., 2015). The protein sequence analysis of the subunit c revealed that the accumulated subunit c had been processed into the inner mitochondrial membrane prior to accumulation, indicated by the cleavage of the mitochondrial import sequence (Palmer et al., 1992, Palmer, 2015).

Subsequently, the subunit c of the mitochondrial ATP synthase was confirmed as the major protein stored in other animals and human NCLs for CLN2, CLN3, CLN5, CLN6, CLN7 and CLN8 (Animal models: Fearnley et al., 1990; Martinus et al., 1991; Jolly et al., 1994; Pardos et al., 1994; Palmer et al., 1997; Url et al., 2001; Cook et al., 2002; Katz et al., 2005; Melville et al., 2005; Frugier et al., 2008; Human NCLs: Palmer et al., 1989a, 1989b, 1992; Hall et al., 1991; Kominami et al., 1992; Kida et al., 1993; Tyynelä et al., 1997; Herva et al., 2000; Palmer, 2015). These show that the storage of the subunit c is a norm in most forms of NCLs and suggest that a common pathological pathway, where different NCLs proteins play a part, underlies the pathological mechanisms of NCLs (Palmer, 2015). In the CLN1 and CLN10 forms, the main storage materials are the sphingolipid activator proteins (SAPs) A and D, which are small heat-stable glycoproteins required for the hydrolysis of sphingolipids in lysosomes (Mehl & Jatzkewitz, 1964; O’Brien & Kishimoto, 1991; Tyynelä et al., 1993; Siintola et al., 2006).

As mentioned earlier, the burden of the storage bodies is not a direct cause of neuronal death (1.3.3). Moreover, the accumulation of the storage bodies occurs in nearly all cells of NCL patients, suggesting the accumulation is not damaging and is a separate consequence of the genetic lesion (Palmer et al., 2002; 2015).

In spite of mounting evidence against the pro-oxidation hypothesis, pro-oxidation is still widely accepted as one of the driving mechanisms for NCLs. The underlying rationale is derived from the proposed oxidative stress that underlies the lipofuscin accumulation in
cellular aging and this oxidative stress is thought to be particularly damaging to post-mitotic cells (Brunk & Terman, 2002; Ottis et al, 2012). Genetic mutations in NCL proteins cause metabolic disruption in the cells and consequently lead to accumulation of storage bodies in lysosomes. The burden of the storage bodies leads to mitochondrial dysfunction, which subsequently aggravates the cumulative process in lysosomes and increases the oxidative stress in cells (Seehafer & Pearce, 2006). The oxidative stress was thought to be facilitated by the presence of transitional metals in lysosomes and to cause mitochondrial dysfunction (Brunk & Terman, 2002; Seehafer & Pearce, 2006). The mitochondrial dysfunction was proposed to cause apoptotic signalling, glutamate excitotoxicity in neurons and oxidative damage to protein, DNA and lipid (Section 1.6, White & Reynolds, 1996; Lemasters et al, 1998; Schapira, 1998; Van Laar et al, 2015). Unfortunately, this hypothesis has largely disregarded the evidence of specific protein storage and has been simply based on possible consequential events of a general metabolic defect (Seehafer & Pearce, 2006). Efforts so far have yielded scant evidence of oxidative stress in NCLs.

3.1.2 ROS, MnSOD and HO-1

One of the major pathogenic mechanisms in NCL is postulated to be oxidative stress caused by the leakage of superoxide anion (O$_2^-$) from dysfunctional mitochondria under pathological conditions (Turrens, 2003). ROS refers to the chemical species with one unpaired electron derived from molecular oxygen. O$_2^-$ is the product of partially reduced molecular oxygen and also a mediator and the precursor of other ROS in oxidative reactions. Spontaneous dismutation/disproportionation of O$_2^-$ occurs when small quantities of hydroperoxyl (HO$_2$) is present in equilibrium in aqueous solution (Bielski & Allen, 1977):

$$\text{HO}_2 + \text{HO}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad (1)$$

$$\text{HO}_2 + \text{O}_2^- + \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad (2)$$

On the other hand, dismutation of O$_2^-$ catalysed by superoxide dismutase to form hydrogen peroxide occurs as follows:

$$2\text{O}_2^- + \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$
The $\text{H}_2\text{O}_2$ in turn may be fully reduced to water by catalases, glutathione peroxidases, and peroxiredoxins or partially reduced to hydroxyl radicals in the presence of chelatable iron as well as $\text{O}_2^-$ (Halliwell, 1978).

The radical character observed for $\text{O}_2^-$ has been over-emphasised and it is known not to be a super radical chemical (Sawyer & Valentine, 1981). It is believed the derived products such as $\text{HO}_2$ and $\text{H}_2\text{O}_2$ are more reactive than $\text{O}_2^-$ (Bielski et al, 1983; Forman & Thomas, 1986). $\text{O}_2^-$ most frequently acts as a reducing agent and thus is unlikely to cause cellular damage by oxidative attack (Ross & Ross, 1977). $\text{HO}_2$ is thought to initiate a lipid peroxidation chain reaction whereas $\text{H}_2\text{O}_2$ reacts with nitric oxide (NO) to produce peroxynitrite (ONOO$^-$) (Bielski et al, 1983; Halliwell & Chirico, 1993; Moncada & Erusalimsky, 2002) but there is lack of in vivo evidence to support the notion (Section 3.1.1). In addition, the hydroxyl radical formation can be prevented by just small amounts of catalases or superoxide dismutases (Halliwell, 1978), indicating efficient removal of $\text{O}_2^-$ by these enzymes and these enzymes can be tools for testing physiological processes for the involvement of $\text{O}_2^-$.

Superoxide dismutases (SODs) efficiently eliminate $\text{O}_2^-$, and form a synergistic relationship with catalases and peroxidases for a defensive mechanism against $\text{O}_2^-$ and $\text{O}_2^-$ derivatives. $\text{O}_2^-$ inactivates catalases and Haem-containing peroxidases by converting these enzymes to the poorly active form by oxidation of iron in Haem. SODs protect catalases and peroxidases against this inactivation. At the same time, $\text{H}_2\text{O}_2$ inactivates superoxide dismutases and catalases or peroxidases prevent this (Fridovich, 1983).

MnSOD (Manganese superoxide dismutase) resides in the lumen of the mitochondrial matrix and functions to convert $\text{O}_2^-$ to $\text{H}_2\text{O}_2$, which is then converted to water by catalases and glutathione peroxidases (Sayre et al, 1999). SOD1 (Cu-Zn SOD), and SOD3 (extracellular SOD; ECSOD; incorporating either copper or zinc) are a family of enzymes that catalyse the dismutation of $\text{O}_2^-$, which was proposed to be produced by phagocytosing neutrophils as part of the bactericidal process (Babior et al, 1973). SODs were subsequently proposed to be protective of the neutrophil-generated superoxide (Salin & McCord, 1975). SOD1 encoded Cu-Zn SOD is primarily located in the cytoplasm of neurons and mutations of SOD1 cause amyotrophic lateral sclerosis (ALS), which is a neurodegenerative disease associated with the relative selectivity of motor neuron cell death (Haidet-Phillips & Maragakis, 2015). The
overlapping roles and the abundance of the SODs in brain suggest an active defensive system against ROS.

In a preliminary CLN6 study, MnSOD expression was reported to be significantly increased in CLN6 affected human fibroblasts and in the brains of CLN6 affected sheep (Heine *et al*, 2003). Elevated MnSOD were also reported in PPT1-knockout mice neurospheres from a murine model of INCL (Kim *et al*, 2006b). Increased MnSOD expression was suggested to be a response to oxidative stress or to activation of glial cytokine production (Heine *et al*, 2003; Kim *et al*, 2006b).

The haem oxygenases (HOs) are claimed to be sensors of cellular oxidative stress (Schipper *et al*, 2009a; b). These enzymes are located in the endoplasmic reticulum (ER) and degrade haem to carbon monoxide (CO), free ferrous ions (Fe$^{2+}$) and biliverdin, which is further reduced by biliverdin reductase A (BVA) to form bilirubin (BR, bile pigment), in the presence of oxygen and NADPH cytochrome P-450 reductase. Two haem oxygenases, HO-1 and 2, have been described to be primarily involved in haem catabolism and erythrocyte turnover in the spleen, liver and bone marrow (Ryter *et al*, 2006). Neurotoxicity caused by HO-1 over-expression has been associated with hyperbilirubinemia (also known as kernicterus in the brain), iron deposition, oxidative stress and mitochondrial injury (Qato & Maines, 1985; Beal *et al*, 1996; Schipper *et al*, 1998; Sayre *et al*, 2001). The accumulation of metals in cells is thought to make cells more vulnerable to the ROS and result in oxidative stress. This has been suggested to be part of the pathogenesis of AD, PD and NCL (Smith *et al*, 2009b; Schipper *et al*, 2009a; Van den Berge *et al*, 2012; Grubman *et al*, 2014). The pro-oxidant property of HO-1 is theoretically sound as the free ferrous ion (Fe$^{2+}$) is a primary reactant in the Fenton reaction, where it reacts with hydrogen peroxide to form a reactive hydroxyl radical, a ferric ion and a proton (Fenton, 1894). The same theory has been applied to most studies that describe transitional metal-provoked oxidative stress. Nevertheless, there is a lack of evidence that it occurs *in vivo* (Section 3.1.1.)

The anti-oxidative role of HO-1 has been suggested to be associated with bilirubin (Stocker *et al*, 1987; Doré *et al*, 1999). Gene knock-out studies of HO-1 showed cells became more susceptible to oxidative stress (Poss *et al*, 1997; Bishop *et al*, 2004). However, the anti-oxidative role of bilirubin only took place when bilirubin bound to albumin (Stocker *et al*, 1987).
1987; Doré et al, 1999), which is not likely to occur naturally within the CNS unless the blood brain barrier is severely breached.

Overall, the oxidative stress theory is not supported by solid evidence.

3.1.3 Neuroinflammation and ROS

A central role of neuroinflammation in NCLs has been well established in systematic studies of glial activation in the ovine model of CLN6, which showed gliosis began perinatally, proceeded regionally, and preceded neurodegeneration (Oswald et al, 2005; Kay et al, 2006). Glial cells function as both the primary source and the target of many cytokines. They also release both neuroprotective and neurotoxic substances in response to different cytokines (Raivich et al, 1999; Du & Dreyfus, 2002).

Enzymatic production of ROS appears to be important in specialised cells. Phagocytes, such as neutrophils and macrophages, produce O$_2^-$ and H$_2$O$_2$ by NADPH oxidase (NOX or PHOX in microglia) in which electrons are transferred from NADPH to O$_2$ (Mander & Brown, 2005). This reaction occurs only when phagocytes are stimulated or during transformation of phagocytes, for phagocytic, secretory and respiratory burst capacities, before stimulation (Babior et al, 1973; Forman & Thomas, 1986). Activated microglial cells have also been suggested to have similar features in production of O$_2^-$ (Section 1.6.1.2).

Activated murine microglial cells, in vitro and in vivo, were found to synthesise iNOS which is inducible by a range of cytokines (Dringen, 2005; Saha & Pahan, 2006; Pautz et al, 2010). iNOS was reported to be increased in the brain of PD patients (Hunot et al, 1996; Reviewed in Knott et al, 2000; Hirsch et al, 2012). Elevated concentrations of reactive nitrogen species have been associated with elevated iNOS expression in a CLN3 mouse model (Herrmann et al, 2008). Neurotoxicity of iNOS-derived nitrogen oxide (NO) was reported to inhibit mitochondrial respiration in neurons (Bal-Price & Brown, 2001, Choi et al, 2009), and result in protein sulphhydryl oxidation (Radi et al, 1991), nitration of proteins, nitrosylation of nucleic acids and breakage of DNA strands (Wink et al, 1991). An alternative mechanism suggested by Mander & Brown (2005), is that the dual activation of iNOS (NO production) and PHOX (O$_2^-$/H$_2$O$_2$ production) in microglial cells can lead to a high production of peroxynitrite subsequently causing neuronal death. However, induction of iNOS in inflammatory cells (monocytes and macrophages) was found to be species-dependent; human, goat, pig and rabbit expressed little iNOS and did not generate detectable NO, in contrast to mouse and
cow (Jungi et al, 1996). A recent study showed that the human iNOS gene is epigenetically silenced (Gross et al, 2014).

MnSOD expression has been associated with neuroinflammation. A range of pro-inflammatory cytokines have been suggested to modulate MnSOD gene transcription. These cytokines are common signalling molecules facilitating an inflammatory response and neurodegeneration (Merrill & Benveniste, 1996). Such cytokines include interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-4 (IL-4) and interleukin-6 (IL-6) (Wong et al, 1988; Wong et al, 1989; Visner et al, 1990; Dougall et al, 1991; Harris et al, 1991).

HO-1 expression is inducible by a range of pro-oxidant (eg. NO) and other stressors, such as pro-inflammatory cytokines (TNF-α, IL-1β), and by glial activation in neurodegenerative diseases such as PD and AD (Kutty et al, 1994a; Chen et al, 2000; McGeer & McGeer, 1995; Rieder et al, 2004). A neuroprotective role for HO-1 has been suggested in a number of cell culture and brain injury studies (Dwyer et al, 1995; Manganaro et al, 1995; Fukuda et al, 1996; Snyder et al, 1998, Chen et al, 2000; Takeda et al, 2000; Huang et al, 2005; Lin et al, 2007). However, the protective mechanism of HO-1 remains elusive.

3.1.4 The mitochondrial marker cytochrome oxidase subunit IV

Mitochondria are the primary site for eukaryotic oxidative metabolism. Mitochondria contain essential enzymes for the Krebs cycle, fatty acid oxidation, electron transport and oxidative phosphorylation. Oxidative phosphorylation is propelled by the thermodynamic efficiency of electron transport, which is facilitated by the redox protein complexes residing in the mitochondrial inner membrane, resulting in reduction of molecular oxygen to water and translocation of protons from the mitochondrial matrix to the mitochondrial intermembrane space. Translocation of the protons creates a proton gradient (pH gradient) for the electrical potential, termed the proton motive force (pmf), which drives the proton-pumping ATP synthase to generate ATP by the phosphorylation of ADP.

The cytochrome c oxidase complex (COX) is the terminal enzyme of electron transport chain and is responsible for the reduction of oxygen to water and translocation of protons. The mammalian mitochondrial cytochrome c oxidase complex consists of 13 subunits, forming a protein complex with a molecular weight of 410kDa (Khalimonchuk & Rödel, 2005). The COX contains four redox centers, including low spin haem a, high spin haem a₃, a copper ion Cu₆
and a CuA (Khalimonchuk & Rödel, 2005). Four electrons from 4 consecutive cytochrome c, 4 substrate protons and one molecular oxygen go through catalytic cycle in the binuclear site (heme a₃, the copper ion Cu₈ and a tyrosine) in the cytochrom c oxidase, resulting in reduction of one molecular oxygen into 2 water molecules and translocation of 4 protons (Wikström, 2012). The cytochrome c oxidase is driven by the energy gradient of electron transport chain and is a proton pump (2H⁺/electron) that contribute to the generation of pmf (Wikström, 2012).

Subunit IV of COX is a well-established marker for mitochondria. It has been reported to act in an early stage of the complex assembly on mitochondria and has shown to co-localise with MnSOD in fibroblasts of the CLN6 patients (Heine et al, 2003; Khalimonchuk & Rödel, 2005). It will be used to demonstrate the correlation between mitochondria and MnSOD.

### 3.1.5 Objectives of the experiments

As described above, previous studies have measured MnSOD protein up-regulation in NCLs (Heine et al, 2003; Kim et al, 2006b; Benedict et al, 2007). The current study aimed to investigate changes of expression in the gene, SOD2, and the associated MnSOD protein at multiple timepoints throughout disease progression to understand the possible correlation between gene and protein expression, as well as determine the critical time of activation. Changes of expression in the genes of iNOS, and HO-1 could also be indicators for oxidative stress in NCLs, since they have been proposed to cause oxidative stress in neurodegeneration (Section 3.1.3).

### 3.2 Materials and methods

#### 3.2.1 Animals

The history, maintenance, genotyping and breeding of the CLN6 ovine model was described in Chapter 2. Sagittal sections from CLN6 affected animals aged 2, 6, 9, 18 and 24 months (n=2 at each timepoint) and age-matched control sheep were immunostained for expression of MnSOD and COX IV. Coronal sections of CLN6 affected animals and age-matched controls at 24 months (n=3) were used to detect the presence and localisation of MnSOD and COX IV. Gene expression of SOD2, iNOS and HO-1 was measured by quantitative PCR as described in Chapter 2.
3.2.2 Immunohistochemical staining for MnSOD and COX IV

Sequential sections were stained for MnSOD with rabbit anti-cow polyclonal MnSOD (1:1000, Abcam, UK) and cytochrome oxidase subunit IV with mouse anti-cow monoclonal COX IV, (1:1000, Invitrogen). Adjacent sections were stained for COX IV and MnSOD. Methods are described in Chapter 2.

3.2.3 RNA isolation and cDNA synthesis

Methods of RNA isolation and single-stranded complementary DNA (cDNA) synthesis are described in Chapter 2.

3.2.4 Primer design and plasmid generation

mRNA specific primer designs were based on ovine or bovine sequences publicly available at NCBI. The online primer design tool BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was utilised for the selection of primers. Optimisation of thermal conditions for selected primers for PCR was as described in Chapter 2. Primer pairs used and optimal annealing temperatures and expected product size are summarised in Table 3.1. Plasmid generation was as described in Chapter 2.
<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>$T_A$ ( ^\circ \text{C} )</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine iNOS</td>
<td>AF223942.1</td>
<td>57 ( ^\circ \text{C} )</td>
<td>External</td>
<td>510</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F- CCC CGT GTT CCA CCA GGA AAT GC</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>R- TGG TCG ATG TCG TGA GCA AAA GCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Internal</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F- GGA GGC CCC AGA GAA GAG AGA TT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R- ACC TTG GGG TTG AAG GCA CAG C</td>
<td></td>
</tr>
<tr>
<td>Ovine MnSOD</td>
<td>GQ221055.1</td>
<td>55 ( ^\circ \text{C} )</td>
<td>External</td>
<td>509</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F- ACG GTG GGG GCC ATA TCA ATC AC</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>R- AGC CAC GCT CAG AAA CAC TAC AAC</td>
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<td></td>
<td></td>
<td></td>
<td>Internal</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F- TTC CGG TTG GGG TTG GCT TGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R- TGC AAG CTG TGT ATC GTG CAG T</td>
<td></td>
</tr>
<tr>
<td>Bovine HMOX1</td>
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<td>External</td>
<td>498</td>
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<td>R- TCA GCA ATC AAG GCC ACA GT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Internal</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>F- AGC CTG AGA CAT CTC CAC CA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R- AAG GGA GAC CTC AGA CCC AA</td>
<td></td>
</tr>
</tbody>
</table>

* Annealing temperature
3.2.5 Quantitative RT-PCR

Thermal conditions for internal primers were verified and optimised by end-point PCR. Quantitative PCR (qPCR) was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) real-time PCR machine and appropriate internal primers in 25μl reactions containing 1μl of cDNA sample (equivalent to 22.5ng/μl mRNA), 12.5μl iQ SYBRGreen Supermix (Bio-Rad), 9.5μl H₂O and 0.2μM of the primers. The thermal cycle conditions were consistent except for adjustments to the annealing temperatures according to specific primer performances (Table 3.1). Detailed information on quality analysis of gene copy numbers and normalisation are described in Chapter 2.

3.2.6 Statistical analysis

Descriptions of statistical analyses were consistent throughout this study (see Chapter 2). Probability values of P < 0.05 between genotypes and ages were considered statistically significant. Levels of significance are indicated as *, P < 0.05;**, P < 0.01.

3.2.7 Microscopy and image analysis

Digital images of MnSOD and COX IV stained sections were acquired as described in Chapter 2. Numbers of immunoreactive cells were estimated using ImageJ (NIH, USA). The sectional area was kept consistent for all images. Collected images were subjected to conversion sequentially to 8-bit, then binary images, prior to using the de-speckle (for background) and watershed (for any merging particles) functions respectively. Noise arising from the staining background was further discriminated by setting up pixels of particles (3-infinity) and circularity (0.3-1.00 μm). Copies of the images were made, and all counted particles were highlighted and labelled. These images were compared to the unprocessed images to ensure that the image processing did not bias the data. Adjustments were then made to images to find the most appropriate setting parameters for determining accurate cell counts. A summary page was generated by the program to display the cell counts. The final cell counts of affected animals were presented as the percentages of the overall cell numbers in controls.
3.3 Results

3.3.1 Immunohistochemistry

Immunohistochemical staining of MnSOD was performed in coronal sections of cerebellum in the CLN6 affected animals and age-matched controls at 24 months (Fig 3.1). Adjacent sections were immunostained for COX IV. Meanwhile, the same immunohistochemical procedure was performed on sagittal sections of CLN6 affected cerebrum and age-matched control sections at 2, 6, 9, 18 and 24 months. The immuno-signal was more intense for MnSOD than for COX IV in almost all the brain regions, except in the deep cortical layer (Fig 3.6; 3.7). Sections stained for MnSOD and visualised using DAB were compared with the adjacent sections stained for COX IV. Positive staining was observed primarily in cells with neuronal morphology and the staining was mainly in the cytoplasm. Glial cells did not react to MnSOD and COX IV antibodies. The immunoreactivity of COX IV paralleled the immunoreactivity of MnSOD in the cerebral cortex, cerebellum and brain stem in both normal and affected animals. The cytoarchitecture of the cerebella and the brainstem of the affected sheep were well preserved, even at advanced disease.
Figure 3.1  Immunostaining for COX IV in the major nuclei of the sheep coronal section through the medulla and cerebellum. F- flocculus, P- paraflocculus, VS-vermis superficial folia, VM – vermis mid folia, VD – vermis deep folia, IC – interposed cerebellar nucleus, MC - medial cerebellar nucleus, LC – lateral cerebellar nucleus, LV- lateral vestibular nucleus, MV – medial vestibular nucleus, SV – spinal vestibular nucleus, DC – dorsal cochlear nucleus, ST - spinal trigeminal nucleus, Gi –gigantocellular reticular nucleus, FN - facial nucleus, R – raphe nucleus, RF – reticular formation, the major nuclei were identified according to the sources of Stockx et al, 2007, and https://www.msu.edu/~brains/brains/sheep/index.html.
### 3.3.1.1 Cerebellum

There was no difference in immunoreactivities of MnSOD and COX IV between the affected and control cerebella (Fig 3.2). Immunostaining for MnSOD appeared to be more prominent in the cerebellum than the immunostaining for COX IV but a similar cellular staining pattern in cells was observed for MnSOD and COX IV. Moderate immunostaining for MnSOD and COX IV was found in some neurons in the molecular and granular layers. The Purkinje cells showed strong cytoplasmic immunoreactivity.

![Figure 3.2 MnSOD and COX IV immunostaining of control and CLN6 affected cerebellar cortex, normal (A, C) and CLN6 affected (B, D). Scale bar represents 100µm.](image)

### 3.3.1.2 Brain stem nuclei

Neurons in the medulla and brainstem were immuno-positive for both MnSOD and COX IV, with no immunoreactive variation between nuclei or genotypes (Fig 3.3). Intense MnSOD and COX VI immunopositivity was localised adjacent to the cell nucleus, and was more diffusive towards the cell periphery and dendritic processes. The burden of storage bodies in
some affected cells resulted in ballooned cell bodies with associated punctate peripheral staining, although this was not a prominent feature in the affected vestibular neurons or other large neurons in the brainstem, such as the Raphe nuclei. (Fig 3.3 E, F, G, H).
Figure 3.3 Comparative MnSOD (A, B, E, F) and COX IV (C, D, G, H) immunostaining of large neurons in vestibular and Raphe nuclei in control and CLN6 affected sheep. The immunostaining intensity did not vary between control and affected animals. Storage bodies altered the peripheral staining of some affected cells, such that they exhibited a punctate appearance as indicated by red arrow-heads. Higher magnifications (the boxed neurons) show specific immunostaining of the respective antibodies. Scale bar represents 500µm.
3.3.1.3 Cerebral cortex

In contrast to a lack of change of immunoreactivity of MnSOD in cerebella and brainstem between the controls and the affected, the immunoreactivity for MnSOD appeared to change with age in the affected cerebral cortices, as a darker band of staining was observed at 18 and 24 months (Fig 3.4). Although not shown, the macroscopic COX IV staining shared a similar trend and was similarly distributed (Fig 3.5).

No immunoreactivity for either MnSOD or COX IV was observed in cells with glial morphology. Not all types of neurons were immunoreactive to MnSOD and COX IV. Positive staining was found in the medium to large size cells with the morphology of pyramidal neurons (Fig 3.5 A; B; Fig 3.6; 3.7). Some were found that morphologically resembled spindle neurons (Fig 3.5 A; B; Fig 3.6; 3.7). The definition for cortical layers was based on the distribution of the pyramidal neurons (Fig 3.5 A; B). MnSOD and COX IV immuno-reactive cells were primarily located throughout layers II to VI and showed heterogeneous expression between the cell types in controls (Fig 3.5 A; B; Fig 3.6; 3.7). It is important to note that these MnSOD and COX IV immunepositive cells were distributed sparsely and unevenly across the cortical layers II to VI in the controls (Fig 3.5 A; B). They appeared to become densely packed at the age of 18 and 24 months in the affected cortices when cortical thinning was evident, leading to an appearance of an increased number of immunoreactive cells (Fig 3.5 A; B). This reflected in the darker band seen in Fig 3.4. However, the intensity of immunoreactivity in the cells did not differ between the affected and control brains (Fig 3.6; 3.7), indicating the abundance of MnSOD and COX IV did not change in these cells.

To confirm if all the immunoreactive cells for MnSOD and COX IV were preserved in the affected brains, these cells were counted and normalised to the number of immunoreactive cells in the controls. In this way, the shrinkage of the surface area in the affected animals was corrected for and the change in MnSOD and COX IV immunoreactive cells could be compared. The cell counts showed number of cells stained for MnSOD decreased significantly at 18 months, and more dramatically at 24 months, as did those immunoreactive to COX IV (Table 3.2). This means there was a disease-correlated reduction of MnSOD and COX IV immunoreactive cells. Since COX IV is a known mitochondrial marker and MnSOD is localised to mitochondria, the mitochondrial number were reduced in the affected cortices.
Table 3.2  Estimation of the reduction in MnSOD and COX IV immunoreactive cells in the CLN6 affected sheep brain at 18 and 24 months. Quantitative cell counts are presented as % of cell in comparison to the controls.

<table>
<thead>
<tr>
<th>Genes</th>
<th>MnSOD</th>
<th></th>
<th>COX IV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/Brain Regions</td>
<td>18m</td>
<td>24m</td>
<td>18m</td>
<td>24m</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>65</td>
<td>31</td>
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<td>27</td>
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<td>Parietal cortex</td>
<td>60</td>
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<td>54</td>
<td>26</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>56</td>
<td>21</td>
<td>52</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 3.4  Immunoreactivity of MnSOD in sagittal sections from the affected animals at 2, 6, 12, 18 and 24 months of age, showing little variation in MnSOD immunoreactivity with the progression of disease from 2 to 18 months. Sections at 24 months of age appear to have a slight increased staining in the parietal and occipital cortex, probably arising from higher background staining. Subcortical nuclei stained for MnSOD remained consistent between genotypes and ages. Boxed areas are presented at a higher magnification in Figure 3.5.
Figure 3.5 Staining of MnSOD and COX IV in the control and CLN6 affected cortices. (A) Immunostaining of MnSOD at 24 months. The parietal cortex of the control animal at 18 month is representative of a number of different cortical regions and ages. Cortical atrophy was pronounced in the affected cortices. (B) Immunostaining of COX IV in affected animals at age of 24 months. The dark lines mark the borders between cortical layers I/II, layers IV/V, and layers VI and the white matter. Magnification is 100 times.
Figure 3.6  Neuronal staining of MnSOD in cortical layer V at 18 months. Cells immunoreactive to MnSOD had the morphology of pyramidal neurons (indicated by the yellow arrowheads). Cell bodies were slightly enlarged in affected animals. Expression was heterogeneous between cells. Scale bar represents 100 µm.

Figure 3.7  Neuronal staining of COX IV in cortical layer V at 24 months. Similarly to the cells immunoreactive to MnSOD, cells stained for COX IV had pyramidal morphology (indicated by the yellow arrowhead). Expression was heterogeneous within individual cells and between cells. The large pyramidal neurons like cells, which were sparsely distributed in the normal cortical layer, were compressed at 18 months (Fig 3.5; 3.6) and more so in the affected cortices at 24 months. Scale bar represents 100 µm.
3.3.2 Quantitative PCR of MnSOD, iNOS and HO-1 mRNA expressions in the brains of CLN6 affected and control sheep

MnSOD, iNOS and HO-1 mRNA expressions were estimated by qPCR to determine whether transcription of these genes changed during disease development in the CLN6 affected cortex. The gene expressions (copy numbers) were extrapolated from standard curves, constructed by serial dilutions of cloned templates of known concentrations, and normalised against endogenous reference genes, GADPH and ATPase, to minimise the variations between qPCR runs.

3.3.2.1 SOD2/MnSOD

Restricted maximum likelihood (REML) method variance component analysis with Wald and F statistics indicated no significant differences in SOD2 expression between frontal, parietal and occipital brain regions for all the affected and control samples. Student paired t-test found significant difference in the parietal and occipital region in SOD2 expression between the genotypes at ages 18 and 24 months (Fig 3.8).

A high basal expression of MnSOD in a range from 2×10⁶ to 5×10⁶ copies per µl cDNA (= 22.5ng of RNA) was found in the cortex of control animals at all ages. SOD2 expression only showed a significant increase in the affected animals at the ages of 18 and 24 months in the occipital cortex of approximately 3 and 4 fold (Table 3.3).
Figure 3.8 MnSOD, iNOS and HO-1 mRNA expression in the cortex of CLN6 affected and control animals at 2, 6, 9, 18 and 24 months of age (n=4). *, significant difference (P < 0.05; paired t-test); **, highly significant difference (P < 0.01; paired t-test) compared to the value for control animals of the same age. Values depict the mean ± SEM for frontal, parietal and occipital brain regions for animals at each time point.

Table 3.3 Fold change of SOD2 (MnSOD)

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Fold change in comparison to age-matched controls</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD2</td>
<td>2-9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>4</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3</td>
<td>*</td>
</tr>
</tbody>
</table>

* -, not significantly different; *, <0.05; **, <0.01
3.3.2.2 \textit{iNOS}

No expression of \textit{iNOS} was found in the CLN6 affected animals or the matched controls (Fig 3.8).

3.3.2.3 \textit{HMOX1}

The HO-1 gene was expressed in very low quantities and expression did not vary between regions, genotype or ages (Fig 3.8).

3.4 Discussion

3.4.1 Mitochondrial protein expression and the oxidative stress hypothesis

In the present study, a thorough investigation of MnSOD immunostaining and mitochondrial staining (represented by COX IV immunostaining) was performed in the cerebellum, brainstem, and the cerebral cortex. The similar pattern of immunostaining for MnSOD and the mitochondrial marker COX IV were demonstrated in all the regions in the observed animals, confirming MnSOD expression correlated with the mitochondrial expression.

One needs to bear in mind that MnSOD directly detects any “leakage” of O$_2^-$ from the redox proteins on the electron transport chain due to any oxidative stress. COX on the other hand is not only a catalytic centre for the O$_2$ reduction but also contributes to the proton pumping that transfers part of the free energy of that O$_2$ reduction as a PMF, which is essentially required for ATP production and normal mitochondrial function. These two endogenous markers are able to represent mitochondrial population as well as cellular sensitivity to the change of oxidative state.

Astrocytes were shown to contain little or no mRNA for cytochrome c oxidase (Hevner & Wong-Riley, 1991). Astrocytes may preferentially require energy from anaerobic glycolysis/glycogenolysis thus glia are not immunoreactive to COX IV (Wong-Riley, 1989), although there has been an indication that astrocytes contain mitochondria (Derouiche \textit{et al.}, 2015). It is presumed that a limited amount of MnSOD is produced (below detectable level) by glial cells if oxidative metabolism is minimal in glial cells.

It was noted that the change of MnSOD and COX IV expression was mainly in their distribution in the affected animals and their distribution was clearly regional, age-related
and disease-related. The distribution of MnSOD and COX IV expression did not change in the cerebella and brain stem in the affected animals whereas they changed dramatically in the cerebral cortices. An appearance of slightly darker immunostaining for MnSOD was highlighted the affected cerebral cortices at 18 months and more so at 24 months at a macroscopic observation, but it was not seen in the earlier ages (Fig 3.4). This impression was contributed to the densely packed immunoreactive cells because of the compression of brain layers in neurodegenerative process (Fig 3.5 A, B). Cell counts of the immunoreactive cells revealed a disease-associated reduction of these cells the most reduced in the parietal and occipital cortex and less in the frontal cortex at 18 months (Table 3.2), in line with the regionality pattern of degeneration in a previous study (Oswald et al, 2005).

The morphology of the immunoreactive cells of MnSOD and COX IV also coincides with the previous observation of which neurons remained at end-stage of disease, showing a selective degeneration of cell types in the disease progression. Nissl staining in the cerebral cortex of CLN6 affected sheep revealed pyramidal cells remained in layer V whereas pyramidal cells were largely missing from layer III in terminal disease at 24 months (Oswald, 2004; Oswald et al, 2008). In humans, small cerebral neurons depleted earlier and faster than the larger neurons, such as the pyramidal neurons (Anderson et al, 2013). The immunoreactive cells were mainly medium to large cells with pyramidal neuronal morphology as well as some spindle-like cells in layer II to IV of neocortex in the controls (Fig 3.5; 3.6; 3.7). Mainly pyramidal neuron like cells remained in the affected cortices at 24 months (Fig 3.5). This means that the selective neurodegeneration in these immunoreactive cells is not dependent on the presence of mitochondria.

Oxidative stress is hypothesised to be caused by metabolic stress resulting from mutated NCL genes and more storage bodies accumulating in cells, and that further increases the oxidative stress (Seefer & Pearce, 2006). This would mean that oxidative stress is a global insult and presumably all cells would be affected equally. In contrast to this hypothesis, the immunoreactive cells of MnSOD and COX IV did not degenerate equally but rather regionally only in the cortex, and not in the other brain regions.

In addition, the intensity and pattern of the immunostaining for MnSOD and the mitochondrial marker (COX IV) in cells in the affected animals was similar to that in the controls, indicating no change in MnSOD abundance, nor any signs of abnormal
mitochondrial activity. Therefore, oxidative stress induced by either storage bodies or neuroinflammation does not play a role in cellular survival.

3.4.2 Regional MnSOD expression and sampling error

Immunohistochemistry for MnSOD and COX IV in the brain showed a highly uneven distribution of these mitochondrial markers within each brain region and between brain regions. Neurodegeneration in the affected animals resulted in a greater change in the distribution of these markers in the affected animals, rendering this distribution more uneven. In neurodegenerative regions, the immunoreactive cells were packed to a narrowed area and led to the appearance of an increased number of immunoreactive cells. This would be especially apparent under the microscopic observation which only allows a small confined area to be seen at a time. Also, the degeneration occurs most severely in the parieto-occipital cortical regions in the affected animals thus the phenomenon of the increased immunoreactive cellular density would be more prominent in these neurodegenerative regions. Therefore, observers should be cautious when they are sampling such microscopic areas for investigation. If the observation disregards the anatomical and cytoarchitectural change in neurodegenerative regions and the sampling areas for experiments in the affected animals are not carefully defined, the false conclusion of an increased number of neurons expressing MnSOD could be drawn. This could well be the cause of the observation found in a previous study, in which elevated expression of MnSOD in the CLN6 affected sheep brain was reported (Heine et al, 2003).

3.4.3 MnSOD gene expression

Quantitative PCR showed elevated MnSOD mRNA expression in the parietal and occipital cortex at the age of 18 and 24 months. This elevation likely results from a sampling error. The compression of parieto-occipital layers could lead to more MnSOD-expressing cells in an overall cell population thus resulting in more RNA for MnSOD, which was compared to the MnSOD RNA collected from a control area in which MnSOD-expressing cells were sparsely distributed amongst other non-immunoreactive cells. The immunoreactive staining for MnSOD and mitochondria well illustrate this possibility of sampling error (Section 3.4.2). The same could occur for other genes subjected to investigations using similar approaches.

However, the cerebral cytoarchitecture is little altered in early disease until neurodegeneration initiates at 6 months and becomes evident at 12 months (Oswald et al,
2005; 2008), so the data collected from the earliest timepoints at 2, 6, and 9 months should remain valid for the main objective, that is to determine if oxidative stress is the primary cause of pathogenesis. MnSOD expression was not changed in the early ages, indicating oxidative stress was not activated.

3.4.4 HO-1 and iNOS mRNA expression

The absolute quantification revealed a huge disparity in mRNA copy numbers between MnSOD, HO-1 and iNOS suggesting differences in the biological significance between these tested genes in the brain. It is not surprising MnSOD has a high basal level of expression since neurons are rich with mitochondria and MnSOD is the superoxide scavenger of oxidative phosphorylation (Sayre et al, 1999). HO-1 expression is minimal in healthy conditions and only induced under pathological conditions in the brain, probably due to biochemical redundancy. The HO-1 isoform, HO-2 exhibits identical substrate and the cofactor specificities (Schipper et al, 2009b). Similarly, iNOS has isoforms (nNOS, eNOS) in the brain that perform the same biochemical function.

Expression of iNOS is species-dependent and its expression in humans has been questioned (Albina, 1995; Jungi et al, 1996). Previous reports have shown human, goat, pig and rabbit express little iNOS and did not generate detectable NO when stimulated, in contrast to mouse and cow (Jungi et al, 1996). A recent study revealed that iNOS is epigenetically silenced in human macrophages (Gross et al, 2014). It is speculated that the species-dependent expression of iNOS in macrophages arises from evolutionary pressure as the antimicrobial activity in human monocytes/macrophages is effective without NO production, which could be cytotoxic as well as inhibitive of an adaptive immune response (Albina, 1995). The study of iNOS gene regulation demonstrated that there are multiple modifications for iNOS gene silencing, including CpG methylation, histone modifications for expression suppression, the low abundance of histone modifications for active expression, and closed chromatin rendered inaccessible to transcription factors (Gross et al, 2014). Though the expression of iNOS in microglia in various species has not been addressed, iNOS is not detected in the ovine brain and there is no reason that it should be expressed. The central nervous system (CNS) is vulnerable and immune-privileged. Specialised NOS such as nNOS and eNOS are responsible for NO production for neuronal signalling and vasodilation. It would be redundant to have another NO production system. More importantly, the
immunosuppression, cytotoxicity and inefficiency of iNOS expression are undesirable in the CNS (Albina, 1995).

mRNA expression of iNOS was not found in the ovine brain in the present study, highlighting a species-dependent expression of iNOS. The iNOS-centred mechanism of inflammation and pro-oxidation should be discarded from the neuroinflammatory cascade in ovine and human NCL. The evidence emphasises variant inflammatory responses across species and that large animal models are more suitable than rodent models for the study of inflammation.

The haem oxygenase-1 (HO-1; HMOX-1) is expressed at low quantities in the CLN6 affected brain. HO-1 may not even play an important role in the CNS. The primary role of HO-1 has been suggested to be involved in haem catabolism and erythrocyte turnover (Ryter et al, 2006). High HO activity is found in tissues that degrade senescent red blood cells. The roles of HO are clearly explained in these cells: HO is present in the bone marrow to inhibit cellular differentiation by lowering the intracellular concentration of haem, which is a differentiation factor for haematopoietic stem cells. HO activity is increased significantly in response to increased circulating haemoglobin in the liver parenchyma, kidney and macrophages (Tenhumen et al, 1970; Pimstone et al, 1971a; b; Abraham et al, 1989; 1991). In traumatised brain, the induction of HO-1 in glial cells was thought to be a scavenging response to the metabolic by-products originating from lysed red blood cells (Fukuda et al, 1996; Matz et al, 1996).

Mitochondria are enriched with haemproteins that are potential substrates for HOs. However, a haem-degrading mitochondrial system, that has a higher catalytic activity than the activity of HOs derived from the microsomal fraction, has been suggested (Kutty & Maines, 1987). The haem-degrading mitochondrial system would be more useful for the tight control in turnover of the mitochondrial haemoproteins (Kutty & Maines, 1987). All the cases indicate the role of HOs is probably mainly in degradation of haem of haemoglobin and HO-1 is not likely induced otherwise. Although in vitro studies found induction of HO-1 in response to anti-oxidant treatment, pro-inflammatory stimuli, hormones, and growth factors (Kutty et al, 1994b; Ryter, 2006; Gozzelino et al, 2010), they have not been able to demonstrate a common mechanism. For the oxidative stress studies in the brain, HO-1 expression was assumed to lead to the deposition of iron and subsequently induce the Fenton reaction-provoked oxidative stress (Schipper et al, 2009b). The storage bodies of
ovine CLN6 and lipofuscin in equine thyroid gland, which are enriched with polyunsaturated fatty acids and transitional metals, showed no sign of pro-oxidation and apoptosis (Palmer, 1987; Jolly et al, 2002; Oswald et al, 2005; 2008). In addition to no induction of HO-1, oxidative stress caused by HO-1 in the presence of transitional metals has little credibility.

3.4.5 Conclusion

Immunostaining for MnSOD and mitochondria revealed a problem associated with random sampling. Interpreting the result of a random chosen sample as a global event is not accurate. The complexity of the discrete change in brain regions and within the region in the neurodegenerative process needs to be considered for future sampling for experiments.

MnSOD as an indicator of oxidative stress was not upregulated either in the mRNA or is the protein in the early stages of the disease progression, indicating that the genetic defect in CLN6 does not provoke pro-oxidation, and oxidative stress is not the main cause of glial activation. It is evident that iNOS was not expressed in sheep brain therefore mitochondrial disruption is not likely to have occurred. In addition, the lack of significant elevation of HO-1 gene expression gave no indication of oxidative stress. Overall, oxidative stress does not play a causative role in the neuropathology. The mounting evidence strongly indicates any pharmacological inhibition of oxidative stress may be of limited efficacy.

The species-dependent expression of iNOS highlights a fundamentally different inflammatory pathway between mice and large mammals such as sheep and humans. This emphasises the importance of using large animal models to complement the deficiency of using rodent models.

3.4.6 Postscript

A postscript containing statistical analyses of MnSOD and COX IV immunostaining of more animals at 18 and 24 months is added after completion of this thesis to support the argument in this chapter.
Chapter 4
Molecular dissection of the neuroinflammatory cascade using selective markers

4.1 Neuroinflammation underlies neurodegeneration

The longitudinal investigation of MnSOD expression in the CLN6 affected ovine brains described in previous chapter did not reveal disease causative changes. Although HO-1 mRNA could be detected in all samples, expression was low and did not increase with disease in any region. There was no iNOS mRNA expression in sheep. It is clear that oxidative stress is not a major driving mechanism for neurodegeneration in the ovine CLN6 disease.

Neuroinflammation has also been investigated in NCLs, revealing that neuroinflammation plays a central role in disease pathogenesis (Pontikis et al, 2004; Oswald et al, 2005; Kay et al, 2006; Kielar et al, 2007; Partanen et al, 2008; Macauley et al, 2011; Kuronen et al, 2012). Suppression of inflammation delayed the clinical progress in Sandoff disease knockout mice, suggesting inflammation may have a causative role in pathogenesis in other diseases (Wu et al, 2004). Glial activation in CLN6 affected sheep mirrors a successive involvement of neuronal loss in the different cortical layers and the brain regions, indicating that glial activation precedes neurodegeneration and may be an important mediator in pathogenesis, being either a stress response or the cause of neurodegeneration.

However, the chronic treatment of CLN6 affected sheep with minocycline, a tetracycline with broad anti-inflammatory properties (Kumar et al, 2003; Nikodemova et al, 2007), did not show any effect on suppression of neurodegeneration, despite early administration prior to clinical onset of neurodegeneration and effective drug absorption into the brain (Kay & Palmer, 2013). The result is not unprecedented as minocycline was not beneficial in treating Huntingtons disease (HD) in a Phase III clinical trial (Schwarz et al, 2010). In concurrent studies, the loss of neuronal populations in ovine CLN6 was characterised as progressive, selective, regional and cell- type specific (Oswald et al, 2005; 2008; Kay et al, 2011). These data highlight cellular location and interconnectivity as the major determinants of neuronal survival. Differences in the local molecular cues may account for the regional and selective neuronal vulnerability. Together, these indicate the complexity of neuroinflammatory
mechanisms. Therefore, it is essential to understand the underlying molecular cues that trigger or/and sustain the inflammatory responses. A dissection of the neuroinflammatory cascade is worthwhile to determine the points of lesion in the pathogenesis of NCLs for effective and target-specific drug intervention.

4.2 Cytokine-mediated neuroinflammation

Cytokines are a diverse group of secretory proteins ranging between 8 and 26kDa in size and produced by a wide variety of cell types (Zhao & Schwartz, 1998). Their action is initiated by binding locally to specific cell-surface receptors, in turn activating intracellular second messenger systems (Zhao & Schwartz, 1998). Cytokine expression is associated with a pathophysiological state when damaging stimuli are released (Benveniste, 1992; Allan & Rothwell, 2001).

A preliminary study of selected pro- and anti-inflammatory cytokines by quantitative real-time PCR, revealed early and persistent disease associated up-regulation of the pro- and anti-inflammatory cytokines, TNF-α and IL-1β, and TGF-β and IL-10, respectively. By 24 months IL-1β and TGF-β had increased further while expressions of both IL-10 and TNF-α decreased. The elevated expression of both pro- and anti-inflammatory cytokines correlated with the initiation of neurodegeneration at 4-6 months of age, prior to the clinical manifestations evident at 10-14 months and widespread cortical atrophy and glial activation evident at 18 months (Barry, 2011). The progressive increase of cytokine expression suggests an uncontrolled inflammatory response is underway. However, a lack of significant regional differences in cytokine expression does not correlate with the progressive and regionally specific glial activation (Oswald et al, 2005; Barry, 2011). Moreover, a contrasting pattern between pro- and anti-inflammatory cytokines further emphasises the complexity of the signalling pathways of pathogenesis. Other contributing factors could be involved, such as cellular location and interconnectivity (Oswald et al, 2008; Barry, 2011). Although cytokine expression may be similar in different brain regions, subpopulations of cells and other determining factors may make them more vulnerable to inflammatory-induced decline (Barry, 2011). Furthermore, the contrasting pattern of cytokine expression may be associated with changes in interneuron populations (Barry, 2011).

The previous cytokine profiling was carried out in the CLN6 affected sheep covering a range of ages from the onset of neurodegeneration, at 6 months, up to the advanced disease. The
cytokine expression data needs to be supplemented with data from a younger age. The roles of cytokines in pathogenesis could then be inferred accordingly, whether cytokine expressions are altered at these ages in affected animals and potentially play a central role in induction of glial activation and in ovine CLN6.

4.3 Dissection of neuroinflammatory cascade

A canonical inflammatory pathway is proposed (Fig. 4.1, Glass et al, 2010): Glial stimulators activate transcriptional pathways such as JAK/STAT, NF-κB and MAPK, increasing production of pro-inflammatory cytokines (Raivich et al, 1999; Li & Verma, 2002; Kumar et al, 2003; Brown & Neher, 2010; Minogue et al, 2012). Simultaneously, these stimulators induce the expression of SOCS3, and anti-inflammatory cytokines, which attempt to hamper the activation of signalling pathways, thus suppressing the production of inflammatory cytokines (Baker et al, 2009). Meanwhile, adaptive immune responses could be activated and contribute to the neurodegenerative process (Lim et al, 2007; Weiss et al, 2008; Saha et al, 2012; Groh et al, 2013). Endosomal-lysosomal processing and sorting of neurotrophic factors and their receptors is affected in diseased animals. It is likely to result in the loss of expression of functional proteins or their receptors, thus affecting the expression of the mRNA. Reduction of neurotrophic factors may have a negative effect on neuronal survival (Henderson et al, 2000; Kopan et al, 2004; Blesch, 2006).
Figure 4.1 A proposed neuroinflammatory pathway. Oxidative stress has been eliminated (Chapter 3).

4.3.1 NF-κB

NF-κB (nuclear factor κB) transcription factors play an important role in the regulation of immune and inflammatory responses. NF-κB induction is essential for the expression of a wide variety of immune response genes, such as pro-inflammatory cytokines TNF-α, IL-1 and 6 (Zhang & Ghosh, 2001). NF-κB is expressed ubiquitously in a variety of cell types in the central nervous system and is involved in cell growth, differentiation and adaptive responses in response to extracellular signals in a cell- and stimulus-specific manner (Kaltischmidt et al, 1993; O’Neill & Kaltischmidt, 1997; West et al, 2002). Up-regulation of NF-κB activity above physiological levels has been associated with trauma and ischemia (Clemens et al, 1997; Bethea et al, 1998; Nurmi et al, 2004), and noted in brains of patients with AD and PD (Kaltischmidt et al, 1997; Hunot et al, 1997). However, the role of NF-κB is not well understood, because of its diversity of interactions between family members and with regulatory elements (Pizzi & Spano, 2006). In general, NF-κB is strongly associated with genes related to immune responses (Ghosh et al, 1998). For instance, induction of NF-κB by stress signals, such as pro-inflammatory cytokines IL-1 and TNF-α, would initiate an
autoregulatory loop that continuously generates more IL-1 and TNF-α, thus sustaining chronic inflammation (Kishimoto et al., 1994).

The term NF-κB commonly refers specifically to a p50-p65/RelA heterodimer, one of the most dominantly formed dimers and the major Rel/NF-κB complex in most cells (Kaltschmidt et al., 2005). A precursor protein p105 is encoded and cleaved post-translationally by the proteasome complex to produce a smaller product, known as p50 (Blank et al., 1991). Repression of p105 (NF-κB 1 encoded) by a p105-based inhibitor showed NF-κB activities were broadly inhibited (Fu et al., 2003), suggesting p105 is essential to NF-κB activities and could be a good indicator of NF-κB activities.

4.3.2 JAK2/STAT1/3

The JAK/STAT signalling pathway directly transduces signals for a variety of cytokines as described in the literature review (1.6.6.2). Cytokines diversify their signalling and deliver complex signals to a receiving cell, possibly via the specific JAKs and the formation of homo or heterodimers of STAT proteins (Kisseleva et al., 2002; Delgoffe & Vignali, 2013). JAK2, STAT1 and STAT3 are the most common cytokine signalling transducers discussed, relating to their facilitation of neuroinflammation that is induced by cytokines like TNF-α, IL-1β, IL-6 (Mcgeer & Mcgeer, 2001; 2004). JAK2-STAT3 was thought to mediate expression of IL-6 as well as IL-10 (Riley et al., 1999; Niemand et al., 2003; Walkley, 2009; Nilsson et al., 2010). The same signalling pathway is thought to be involved in the mediation of glial activation in vitro and in vivo models of several neurodegenerative diseases, namely AD, HD and a lysosomal storage disease (Sandhoff disease, SD) (Sriram et al., 2004; Huang et al., 2008; Abo-ouf et al., 2013; Xiong et al., 2014). JAK2- STAT1 is known for playing a part in the type I and type II interferon signalling transduction, which is targeted by virus evasion (Goodbourn et al., 2000; Parisien et al., 2002). Upon activation, STAT1 forms homodimers or heterodimers with STAT2 and initiates transcription of pro-inflammatory mediators (Aaronson & Horvath, 2002). STAT1 was found to be widely distributed in the brain, and primarily changed in neurons upon brain insult (Wang et al., 2002a; Campbell, 2005), suggesting that it is likely to directly mediate inflammatory responses in the neurons. JAK2 is one of the primary upstream kinases that activates STAT1/3 (Aaronson & Horvath, 2002). JAK2-STAT1 was also reported to modulate production of pro-inflammatory cytokines upon glial activation (Gorina et al., 2011; Minogue et al., 2012).
4.3.3 MAPK14

The mitogen-activated protein kinase (MAPK) signalling pathway is a convergent hub for a wide range of external signals. The discovery of the family was associated with the production of the pro-inflammatory cytokines in response to cellular stress (Freshney et al, 1994; Han et al, 1994; Lee et al, 1994; Rouse et al, 1994). p38α is ubiquitously expressed in most cell types and is the best characterised of all (Cuadrado & Nebreda, 2010). The signalling transduction of MAPK were reviewed in Chapter 1 (1.6.6.5).

p38α (MAPK14) and p38β (MAPK11) have highly similar three dimensional structures and 75% identical amino-acid sequences, except for differences in the ATP-pocket, suggesting that they may have overlapping substrate specificities and functional redundancies (Cuadrado & Rousseau, 2007; Patel et al, 2009; Cuadrado & Nebreda, 2010). In the present study, p38α (MAPK14) is the primary MAPK kinase probed. It has been implicated in the chronic neuroinflammation for prolonged overproduction of pro-inflammatory cytokines in AD and was thought to be an early event in the disease progression (Munoz et al, 2007; Bachstetter et al, 2011). Studies showed the up-regulation of pro-inflammatory cytokines such as IL-1β and TNF-α were mediated by activation of p38α and that the inhibition of p38α led to a reduction of the pro-inflammatory cytokines (Munoz et al, 2007; Bachstetter et al, 2011).

4.3.4 SOCS3

The suppressors of cytokine signalling (SOCS) family of cytoplasmic proteins serve as the suppressive regulators to attenuate cytokine signal transduction and act through the inhibition of the JAK/STAT pathway. The introduction of SOCS family is described in Chapter 1.

SOCS3 is one of the major SOCS proteins expressed in the CNS upon an inflammatory response (O'Shea & Murray, 2008). It is suggested to primarily inhibit signalling of the IL-6 family of cytokines. It binds to the receptor gp130 where it inhibits JAKs activity and targets JAKs for degradation via the SOCS box (Lang et al, 2003; Lehmann et al, 2003; Shuai & Liu, 2003; Yasukawa et al 2003; El Kasmi et al, 2006; Boyle et al, 2007; Murray, 2007; Babon et al, 2008). SOCS expression can be stimulated by STATs activation, serving as a negative feedback loop to the cytokine activation. For instance, JAK/STAT3 was inhibited by SOCS3, especially in astrocytes (Croker et al, 2008; Qin et al, 2008). SOCS3 appeared to play a role in
suppression of astrocytic activation, which was provoked by inflammatory cytokines and which produced chemokines that initiated microglial activation and T cell infiltration into the brain (Qin et al, 2008). In microglia, the anti-inflammatory action of IL-10 seemed to relate to the enhanced expression of SOCS3, while in turn attenuating JAK/STAT activation (Lang et al, 2003; Yasukawa et al, 2003; Qin et al, 2006; O’Shea & Murray, 2008). It was also reported to inhibit other cytokine related signalling pathways, such as NF-κB (Baetz et al, 2004).

4.3.5 Neurotrophic factors

Neurotrophic factors promote neuronal survival, support nervous system development, and the maintenance and function of the adult nervous system. Therapeutic use of these factors to intervene in neurodegenerative diseases like AD and PD appeared to show promising results, emphasising the importance of neuronal survival supported by neurotrophic factors in neurodegenerative diseases (Blesch, 2006).

A number of studies have shown that the loss of neurotrophic support for selective neuronal populations may contribute to the pathology of neurodegenerative diseases, and in some circumstances treatment with neurotrophic factors can prevent cell loss. Reduction of BDNF expression was found in nigral neurons and was thought to be related to the selective degeneration of the nigral dopamine neurons of PD (Venero et al, 1994; Mogi et al, 1999; Howells et al, 2000; Porritt et al, 2005). A recent study showed transfection of BDNF into the dopamine neurons had a positive effect on the recovery of the striatal innervation, dendritic spines and motor behaviour of a PD mouse model (Razgado-Hermendez et al, 2015). In AD, BDNF deficiency in the hippocampus was considered part of the pathogenesis and the increased BDNF expression was demonstrated to rescue the hippocampal neuronal loss and the dendritic atrophy (Siegel & Chauhan, 2000; Meng et al, 2013). Depletion of BDNF and TrkB were also found in mice and human brains HD (Zuccato et al, 2008). The AAV vector – mediated gene therapy of BDNF and TrkB showed neuronal protection in an excitotoxic striatal lesion of HD (Connor et al, 2015).

BDNF is highly expressed throughout the brain and is primarily present in neurons (Connor & Gragunow, 1998). There are suggestions that BDNF could be taken up by axon terminals at the site of synthesis and retrogradely transported to the target cell bodies, where its presence has been detected in the endosomal or lysosomal compartments (Wetmore et al, 1991; Mufson et al, 1994). Moreover, pro-BDNF was found to be shuttled from the trans-
Golgi network into secretory granules, where it is cleaved by the pro-hormone convertase 1 (PC1) (Farhadi et al, 2000). Trk signalling is thought to participate in the retrograde vesicle signalling of their associated neurotrophic factors (Miller & Kaplan, 2001). The BDNF-Trk B complex is likely endocytosed and transported to the cell body in vesicles. The vesicles are termed as peripheral early endosomes, where the sorting of the BDNF-Trk B complex takes place. The BDNF-Trk B complex is either recycled, or processed to lysosome for degradation (Section 1.7).

BDNF signalling is compromised in affected neurons in the lysosomal storage disease Niemann-Pick type C, suggesting that the sorting of BDNF neurotrophic receptors is impaired (Henderson et al, 2000). Reduction of BDNF was also reported in Gaucher disease and mucopolysaccharidosis IIIB (Kim et al, 2006a; Villani et al, 2007). Defective processing of neuropeptides has been suggested in CLN2 affected human fibroblasts (Kopan et al, 2004). Meanwhile, CLN6 has been suggested to associate with pre-lysosomal vesicular transport (Fossale et al, 2004; Heine et al, 2004; Metcalf et al, 2008). This means that the sorting and processing of neurotrophic factors could be affected by the defective CLN6, resulting in reduced expression of neurotrophic factors and their receptors and in turn neuronal dysfunction and loss.

### 4.3.6 Lymphocytic infiltration

The blood brain barrier (BBB), formed by the lining of endothelial cells in the cerebral blood vessels, provides a diffusion restraint between the circulating blood and the brain.

Lymphocytic infiltration has been considered part of the disease mechanisms in AD, PD, ALS and NCLs (Lim et al, 2007; Saha et al, 2011; Kannarkat et al, 2013; Evans et al, 2013; Groh et al, 2013; Lynch, 2014). Pro-inflammatory cytokines such as TNF-α and IL-1β released upon glial activation could directly alter the expression of cellular adhesion molecules (CAMs) on the endothelium. The CAMs can bind to receptors on leukocytes, which ultimately infiltrate the CNS (Raine et al, 1990; Shimizu et al, 1991; Barten & Ruddle, 1994; Verbeek et al, 1995; Haraldsen et al, 1996; Wong et al, 1999). However, it is not clear how the infiltrated T cells participate in the pathogenesis of the neurodegeneration. Studies proposed that the infiltrated T cells release pro-inflammatory cytokines that exacerbate neuroinflammation (Saha et al, 2012; Groh et al, 2013; Zhang et al, 2013). The crossing of Ppt1−/− mice (a mice model of infantile NCL/CLN1), with mutants lacking lymphocytes (Rag1−/−, which is crucial for
normal lymphocyte development, Shinkai et al, 1992), showed amelioration of the functional and clinical phenotypes and improved longevity (Groh et al, 2013).

Other mechanisms of BBB disruption have been proposed including the modulation of tight junction proteins by pro-inflammatory cytokines which increases the BBB permeability, and activated metalloproteinases (MMPs) directly contributing to an alteration of the basal lamina (Weiss et al, 2008).

Recent studies in INCL and JNCL mouse models have suggested an alteration of the permeability of the BBB and the infiltration of the active T cells, suggesting the adaptive immune response is involved, along with innate glial activation of the CNS (Lim et al, 2007; Saha et al, 2011). Breaches in the BBB allow the entry of the immune cells and their activation may damage neurons. Possible BBB disruptions can be investigated by using sheep specific antibodies for IgG and lymphocyte subsets expressing CD4 (T helper cells) and CD8 (T cytotoxic cells). These markers were used in previous studies of other NCLs (Lim et al, 2007; Groh et al, 2013).

4.4 Materials and methods

4.4.1 Animals

Details are described in Chapter 2. Tissue from sheep aged 2 months were added to the mRNA study of cytokines. The same animals were used for quantitative PCR of cytokines in the previous study.

4.4.2 RNA isolation and cDNA synthesis

Methods of RNA isolation and single-stranded complementary DNA (cDNA) synthesis are described in detail in Chapter 2.

4.4.3 Primer design and plasmid generation

The method is described in Chapter 2.

Primer pairs were designed to target the sequences shared between the transcript variants of a gene. Purified products generated by primer pairs were sequenced to ensure that the correct products were acquired and sequences of the ovine genes of interest were
constantly updated particularly if the primer designs were based on the bovine sequences. Products of these primers were sequenced and aligned with the updated ovine sequence to confirm the correct targets.

Table 4.1  Primer sequence information and reaction conditions for qPCR of neuroinflammatory markers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence reference in GenBank</th>
<th>Ta*</th>
<th>Primers</th>
<th>Expected product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine JAK2</td>
<td>XM_004004357.1 (transcript variant 1) XM_004004358.1 (transcript variant 2)</td>
<td>55 ºC</td>
<td>External F- TGCCGGTATGACCTCTCTACA R- ATGGGACTTTCACCAGGCTC</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57 ºC</td>
<td>Internal F- GGTTGGCTGTAAGAAGCTCC R- GCCAGCAGCTAAGAAGCTC</td>
<td>130</td>
</tr>
<tr>
<td>Ovine STAT1</td>
<td>NM_001166203.1</td>
<td>55 ºC</td>
<td>External F- CAATGCTTTGCTGATGACCTC R- CTCCGGTTGATGAGCTC</td>
<td>441</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57 ºC</td>
<td>Internal F- CACTGTAGTTGGCAGCTC R- GTTGAAGGTGCGGTCCATA</td>
<td>133</td>
</tr>
<tr>
<td>Ovine STAT3</td>
<td>JF267352.1</td>
<td>57 ºC</td>
<td>External F- CTTCTCCTTGGGCTGACCTC R- TCGGCAGCTCAGTGGATATTG</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57 ºC</td>
<td>Internal F- CAAAGGAGGAGGCATTGCTGCA R- GGCAGGTCAGTGGATATTG</td>
<td>139</td>
</tr>
<tr>
<td>Ovine NF-kB 1</td>
<td>BC153232.1</td>
<td>55 ºC</td>
<td>External F- CTATGACAGCAAAGCCCCCA R- GCCATACGTGGGGAATCCAT</td>
<td>529</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55 ºC</td>
<td>Internal F- CCTCTGTTGCAGCTGAGCTT R- TACCACCCGAAAACCTGTCC</td>
<td>153</td>
</tr>
<tr>
<td>Ovine MAPK14</td>
<td>NM_001142894.1</td>
<td>55 ºC</td>
<td>External F- CGAGCGTTACCAGAACCTGT R- TGTCGAGCCAGTCCGAAATC</td>
<td>459</td>
</tr>
</tbody>
</table>
For the majority of the genes studied in this chapter, home made master mix was used for the quantitative RT-PCR. This contained the optimal concentration of essential reagents, (Invitrogen) including 10X buffer, 3mM MgCl₂, 0.2mM dNTPs, and 10X SYBR green, primers (0.16μM per primer), 0.5U Platinum Taq DNA Polymerase (Invitrogen) and water in a 15μl reaction. Quantitative RT-PCR was performed on an iCycler iQ real-time PCR detection system (Bio-Rad).

The expression of reference genes (GAPDH and ATPase) were determined in the same samples for all the genes and in home made RT-PCR reagents when necessary.

Thermal cycling conditions and methods of data collection are described in Chapter 2.
4.4.5 Statistical analysis

The statistical analyses were kept consistent throughout the study (see Chapter 2). Probability values of P < 0.05 were considered statistically significant between genotypes and ages. Levels of significance are indicated as *, P < 0.05 ; **, P < 0.01.

4.3.6 Western blotting analysis

4.3.6.1 Protein extraction
The frontal, parietal and occipital lobes of the most medial slab of each 6, 9 and 18 month old CLN6 affected brains were dissected for SOCS3 protein detection. SOCS3 is a cytoplasmic protein and readily soluble in detergent free buffer (Park et al, 2014). Total soluble protein fractions were extracted by homogenising 300mg of grey matter from the frontal, parietal and occipital corticies in 1.2ml of extraction buffer (20mM Tris-HCl, pH7.5; 1mM EDTA, 5mM MgCl₂) containing phosphatase inhibitors (0.1 NaF, 1mM β- glycerol phosphate, 1mM Na₃VO₄) and protease inhibitor cocktails (Roche Diagnostics, IN, USA), using a D130 homogeniser (LabServ, Ireland). Protein storage and concentration measurements are as described in Chapter 2.

4.3.6.2 Protein electrophoresis and transfer
As described in Chapter 2.

4.3.6.3 Western blotting analysis
Western blotting was also used to monitor expression of proteins in the brain homogenates from selected regions at different ages. The optimal antibody concentration was initially determined on protein extracted from an 18 month-old affected animal and a healthy counterpart. The specificity of immunoreactivity of the antibody was observed by a band at the expected molecular weight. Mouse anti-human GAPDH (1:25000, monoclonal, Bio-Rad) was selected as the loading control. The concentration of rabbit anti-human SOCS3 primary antibody (1:1000, polyclonal, Abcam) was diluted in 3% blocking buffer (3% bovine albumin serum in TBST) and relevant secondary antibodies were applied. Immunoblotting, washing and developing of the blots were as per described in Chapter 2.

Initial immunoblotting for SOCS3 showed specificity for SOCS3 and did not produce spurious bands that would interfere with GAPDH detection. Therefore blots stained for SOCS3 were
washed in TBST, then incubated with GAPDH antibodies followed by biotinylated secondary antibodies and ExtrAvidin peroxidase.

4.3.7 **Immunohistochemistry**

4.3.7.1 **Tissue collection and processing**

Brain tissue from affected sheep aged 2, 6 and 24 months and age matched controls were used. Methods of perfusion fixation and tissue sectioning are elaborated in Chapter 2.

Mesenteric lymph nodes from animals infected with the parasitic gastrointestinal nematode *Trichostrongylus colubriformis* were paraffin wax-embedded and 5µm sections serially cut from each block and mounted on superfrost plus glass slides (Gribbles Veterinary Pathology, Christchurch, New Zealand). These lymph node samples were kindly donated by Dr Andy Greer and Andrea Hogan (Faculty of Agriculture and Life Sciences, Lincoln University), and were shown to have high CD4+, CD8+ T cell counts by flow cytometry. These lymph nodes were processed for use as positive control tissues for CD4+, CD8+ expression.

4.3.7.2 **Immunohistochemistry**

Antibodies used were mouse anti-ovine CD4 (1:50, monoclonal, AbD Serotec, USA), mouse anti-ovine CD8 (1:50, monoclonal, AbD Serotec) and rabbit anti-ovine IgG (1:500, polyclonal, AbD Serotec). The CD4 and CD8 stainings were performed on mesenteric lymph nodes.

Paraffin embedded lymph node sections were de-waxed and re-hydrated by washes of xylene and a diluted alcohol series: 2 washes in xylene, 5 min per wash; 2 washes in 100% ethanol, 3min per wash followed by 95%, 70% and 50% alcohol washes, 3min per wash, and a final 3min wash in water. Subsequently, the sections were immunostained. All experimental sections were subjected to an antigen retrieval process. The tissue sections were incubated overnight in 0.1M sodium citrate buffer, pH 4.5, at 4°C, then transferred to 6-well plates, containing 10ml of fresh buffer per well, and irradiated in a household microwave oven at 650W for 30s. After cooling to RT, sections were blocked for 30 min with 1% H₂O₂ in PBST to quench endogenous peroxide activity and for 60 min in 15% NGS in PBST to remove non-specific tissue antigens. All steps were followed by three washes in PBST. Sections were then incubated with primary antibodies, overnight, 4°C, diluted in 10% normal goat serum (NGS) in PBST (PBS, pH7.4, containing 0.3% Triton X-100). Negative control brain
sections in which the primary or secondary antibodies were omitted, were included on the same incubation plate.

Secondary and tertiary antibody incubations and colour development were shown as described in Chapter 2.
4.4 Results

4.4.1 Cytokine expressions

![Graphs of cytokine expressions](image)

**Figure 4.2** Quantitative real-time PCR analyses of *IL-1β* (A), *TNF-α* (B), *TGF-β* (C) and *IL-10* (D) mRNA expression, in the brains of affected and control animals at 6, 9, 18 and 24 months of age. Changes in expression between the affected and control animals of the same age were analysed by paired t-tests on log-transformed data. Values depict the mean ± s.e.m. for frontal, parietal and occipital brain regions for animals at each time point compared to the value for control animals of the same age. *, significant difference (P < 0.05; paired t-test); **, highly significant difference (P < 0.01; paired t-test) (2 month data added to data from Barry, 2011).
Pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory (TGF-β, IL-10) cytokine expression from animals aged from 6 to 24 months were analysed and discussed in Barry (2011). In summary of results obtained by Barry (2011), TNF-α, IL-1β, TGF-β and IL-10 expressions increased in the affected animals compared with the controls at all ages analysed, including 6 months, well before clinical disease is evident. However, they followed a different expression patterns with disease progression in terms of copy numbers and fold increases. Steady increases of IL-1β and TGF-β expression were observed while TNF-α and IL-10 expressions increased at 6 months followed by a slight decrease at 9 months and peaked at 18 months. They subsequently reduced but remained significantly high at 24 months (Fig 4.2).

No significant difference in expression was found between any of the cytokines in the frontal, parietal and occipital brain regions of the control samples or between the affected brain regions at any ages. There was a lack of activation of cytokine expression at 2 months.

4.4.2 Gene expressions of transcriptional factors responsible for pro-inflammatory cytokine expressions

Experimental animals, methods, and statistical analyses were consistent with the previous study (Barry, 2011) to provide a consistent map of the neuroinflammatory pathway regulated by the selected mediators.

4.4.2.1 NF-κB1

NF-κB1 was expressed in control animals at all the ages at a low copy number (Fig 4.3). Expression did not vary between regions in either the control or affected animals and the pattern was similar between the control and affected animals at the ages of 2, 6 and 9 months. Significant differences in expression became apparent at 18 months and 24 months (P<0.01). Dramatic increases of 5 fold were observed in the affected animals at 18 months and of 4 fold at 24 months, reaching copy numbers of 3.9x10^4 copy/µl cDNA (1µl cDNA =22.2ng mRNA) at 18 months and 1.7x10^4 copy/µl cDNA at 24 months (Table 4.2). The controls had a mean copy number of 7.9 x10^3 copy/µl cDNA at 18 months and 4.2 x10^3 copy/µl cDNA at 24 months.
Figure 4.3  Quantitative PCR analysis of *NF-κB* 1 mRNA expression with disease progression. Expression of NF-κB 1 in affected animals is similar to control animals at 2, 6 and 9 months then increased significantly at 18 months. Although the expression decreased at 24 months, it remained significantly higher than age matched controls.

Table 4.2  Fold change of *NF-κB1*

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Fold change in comparison with the age-matched controls</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NF-κB</em> 1</td>
<td>2-9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>5</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4</td>
<td>*</td>
</tr>
</tbody>
</table>

*-*, not significantly different; *, <0.05; **, <0.01

4.4.2.2  *MAPK14*

*MAPK14* did not altered dramatically in affected animals with disease progression and demonstrated a similar expression pattern to that in the controls (Fig 4.4). Although expression of *MAPK14* appeared higher in the affected animals compared to the controls from 6 to 24 months, it was only statistically significant (P<0.05) at 18 months. Merely 2 fold
of change was found (control mean, $3.4 \times 10^3$ copy/µl cDNA; affected mean $6.2 \times 10^3$ copy/µl cDNA) at 18 months (Table 4.3).

Results for affected animals were highly variable, so although there was a trend of increase at 6, 9, 18 and 24 months, the results were only significantly higher at 18 months (Fig 4.4).

Figure 4.4  Quantitative PCR analyses of MAPK14 expression with disease progression. MAPK14 expression appeared to slowly increase over the course of disease progression, but the increase only became significant in the affected animal at 18 months.

Table 4.3  Fold change of MAPK14

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Fold change in comparison with the age-matched controls</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPK14</td>
<td>2-9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*-, not significantly different; *, <0.05; **, <0.01
4.4.2.3 JAK2/STAT1/STAT3

The expression of cytokine associated signal transducers, JAK/STAT, did not vary between regions (Fig 4.5). The expression varied between the signal transducers. Basal mRNA expression of JAK2, STAT1 and STAT3 were present in the control animals at all ages with slight fluctuations between the different ages. In the affected animals, although JAK2 expression showed a trend of increase in the affected animals, but the expression was highly variable at the age of 6, 9, 12 and 18 months and only became significant at 2 fold at 18 months (Fig 4.5; Table 4.4, control mean, 5.5x10^3 copy/µl; affected mean, 1.2x10^4 copy/µl).

The expression of STAT1 was not significantly different between affected and control animals at 2 and 6 months but then showed a significant 3 fold increase in the affected animals at 9 months (Table 4.4, control mean, 1.7x10^4 copy/µl cDNA; affected mean, 5.5x10^4 copy/µl cDNA) and a 5 fold change at 18 months (control mean, 1.5x10^4 copy/µl cDNA; affected mean, 7.7x10^4 copy/µl cDNA), and decreased at 24 months (control mean, 9.2x10^3 copy/µl cDNA; affected mean, 3.8x10^4 copy/µl cDNA).

Expression of STAT3 was similar in the control and affected animals at the age of 2 months, after which it became different, from 2 fold at 6 months (control mean, 6.0x10^4 copy/µl cDNA; affected mean, 1.2x10^5 copy/µl cDNA) to 4 fold at 18 months (Table 4.4, control mean, 8.5x10^4 copy/µl cDNA; affected mean, 3.5x10^5 copy/µl cDNA) followed by a decline to 3 fold at 24 months (control mean, 5.9 x10^4 copy/µl cDNA; affected mean, 2.0 x10^5 copy/µl cDNA).
Figure 4.5  Quantitative PCR analysis of JAK2 (A), STAT1 (B) and STAT3 (C) mRNA expression, in the cortex of CLN6 affected and age matched control animals at 2, 6, 9, 18 and 24 months of age. Minimal control expression remained unchanged with age.

A. JAK2 expression in the affected animals remained similar to the expression in the controls at 2 months. The expression in the affected animals increased over controls, but was only significant at 18 months.

B. STAT1 expression in the affected animals increased from 6 months to 18 months and decreased at 24 months. Expression in the control animals was stable and at low copy numbers at all ages.

C. STAT3 expression in affected animals was increased at 6 months, more so at 18 months then declined at 24 months.
Table 4.4  Fold changes of JAK2, STAT1 and STAT3

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Fold change in comparison with the age-matched controls</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK2</td>
<td>2-9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STAT1</td>
<td>2-6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>5</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4</td>
<td>*</td>
</tr>
<tr>
<td>STAT3</td>
<td>2-6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>4</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3</td>
<td>*</td>
</tr>
</tbody>
</table>

*-, not significantly different; *, <0.05; **, <0.01

4.4.3  Gene expression of suppression of pro-inflammatory cytokines

4.4.3.1  Quantitative RT-PCR of SOCS3

Expression of SOCS3 in the affected animals was elevated as early as 2 months of age, increased progressively from 2 to 9 months and multiplied dramatically at 18 months (Fig 4.6). Expression in the affected animals declined sharply at 24 months but remained significantly higher than that in the age-matched controls. The lowest expression level in the affected animal was 360 copy/µl cDNA, whereas the matched control was below 100 copy/µl cDNA, while as many as 5.1x10^4 copy/µl cDNA were found in 18 month-old affected animals.
SOCS3 was expressed very little in the control animals in all the ages and was significantly higher in the affected animals at all ages (Fig 4.6). Expression did not vary between brain regions. The range of up-regulation in the affected animals varied from 5 fold to 74 fold (Table 4.5). It was by far the most dramatically altered gene and the only gene up-regulated prior to 6 months found in the present study.

**Figure 4.6** Quantitative PCR analysis of SOCS3 mRNA expression. SOCS3 expression in the affected animals significantly increased at all ages compared to age-matched controls. Expression was highest at 18 months after which it decreased.

**Table 4.5** Fold change of SOCS3 expression

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Fold change in comparison with the age-matched controls</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS3</td>
<td>2</td>
<td>5</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>14</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>21</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>74</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>64</td>
<td>**</td>
</tr>
</tbody>
</table>

*-, not significantly different; *, <0.05; **, <0.01
4.4.3.2 **Western blottings of expression of SOCS3**

Protein expression of SOCS3 was investigated by Western blotting (Fig 4.7; Table 4.5), which revealed a crisp single band at approximately 27kDa in the affected frontal cortex at 18 months, and the parietal and occipital cortices at 9 and 18 months. No immunoreactive bands were detected in the control brain homogenates. Expression was highest in the affected cortices at 18 months. Some expression was visible in the 9 month-old affected animal.

GAPDH was selected as a protein loading reference for SOCS3 detection. GADPH antibody detected a single immunoreactive band at 37kDa (Fig 4.7). The GAPDH immunoreactive bands in the affected frontal cortex appeared less intense compared to the GAPDH signals from the occipital samples. The GAPDH signals in the parietal cortex were less distinct than those from the other two regions.

![Western Blot Image]

**Figure 4.7** **SOCS3 detection by Western blotting.** Blots of brain homogenates, isolated from frontal, parietal and occipital lobes probed with antibodies against SOCS3. 50μg protein per lane were loaded. GAPDH was the reference for protein loading.
4.4.4 Neurotrophic factor and receptor expression

Figure 4.8 Quantitative PCR analysis of BDNF (A) and Trk B (B) mRNA expression.

A. *BDNF* expression in the affected animals was similar to that in the control animals throughout the disease progression.

B. *Trk B* expression in the affected animals was significantly higher at 18 months. The expression declined but remained significantly higher than the controls at 24 months.
Table 4.6  Fold change of BDNF and Trk B

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Fold change in comparison with the age-matched controls</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>2-24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trk B</td>
<td>2-9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>3</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2</td>
<td>*</td>
</tr>
</tbody>
</table>

*- -, not significantly different; *, <0.05; **, <0.01

BDNF expression did not vary between the brain regions, ages and genotypes (Fig 4.8; Table 4.6).

The expression of the BDNF receptor Trk B in the control sheep was similar in all brain regions of all ages (Fig 4.8; Table 4.5). The mean expression of Trk B in the controls was around 1x10^5 copy/µl cDNA while that BDNF was 5000 copy/µl cDNA and the expression pattern mimicked BDNF expression. In contrast, Trk B expression in the affected animal showed little similarity to that of BDNF. It was not significantly different from the controls at the age of 2, 6 and 9 months, but increased three times more than age-matched controls at the age of 18 months (control mean 1.6x10^5 copy/µl cDNA; affected mean 4.3x10^5 copy/µl). Expression of Trk B decreased from 18 months to 24 months but was still almost twice that in the controls at 24 months.

4.4.5  Blood-brain-barrier breach

A disruption of the BBB would be indicated by IgG deposition within cells in the CNS and infiltration of lymphocytes into the CNS. This can be detected by the immunoreactivities of IgG and cell surface markers of lymphocytes.

4.4.5.1  Immunohistochemical staining of lymphocytic markers

The possibility of BBB breaches in the CLN6 affected brains at advanced stage were investigated by staining for the lymphocytic markers for CD4 (T helper cells) and CD8 (T cytotoxic cells) (Fig 4.9). Lymph nodes with high lymphocytic cells counts from parasite infected sheep were immunostained as positive controls for the lymphocytic markers.
Analyses of these sections revealed high numbers of cells immunoreactive to CD4 and CD8 in lymph nodes infected by parasites (Fig 4.9 B; Fig 4.10 B). No immunoreactive cells were found within the brains (Fig 4.9 D; Fig 4.10 D).
Figure 4.9  Immunohistochemical staining of CD4 in a control lymph node (A) a parasite-infected lymph node(B) a control cortex at 18 months (C) and a CLN6 affected cortex at 18 months (D). Scale bar represents 100μm. The parasite-infected lymph node showed evident cellular staining. Some predicted anatomical structures of lymph were depicted as CA capsule; F follicle; P paracortex; and R reticular fibre.
Figure 4.10  Immunohistochemical staining of CD8 in a control lymph node (A) a parasite-infected lymph node (B) a control cortex at 18 months (C) and a CLN6 affected cortex at 18 months (D). Scale bar represents 100μm. The parasite-infected lymph node showed evident cellular staining. Some predicted anatomical structures of lymph were depicted as AT adipose tissue; CA capsule; F follicle; P paracortex; SS subcapsular sinus; and T trabecula.
4.4.5.2 IgG deposition in the CLN6 affected brain

IgG deposition in the 18-month-old CLN6 affected brains was examined by staining for heavy and light chains of ovine IgG. Perivascular immunoreactivity of IgG was evident in the control and affected brains (Fig 4.11 A, B) and not found in sections omitting the IgG antibody (Fig 4.11 C). No immunoreactivity was found within the CNS.
Figure 4.11  Immunoreactivity of IgG in cortex from the control and affected animals at 18 month. Control cortex at 18 months (A), CLN6 affected cortex at 18 months (B), for a negative control, IgG antibody was omitted in the CLN6 affected section (C).
4.5 Discussion

4.5.1 The cytokine expression profile at 2 months

The selected ages marked the timepoint at which the important pathological features during disease progression (Section 2.1; Fig 2.2). Activated glial cells were evident in the affected sheep brain perinatally, marking an early activation of neuroinflammation (Oswald et al., 2005; Kay et al., 2006). Neurodegeneration, predicted by glial activation and reflected by changes in neuron populations, initiates at 6 months. Clinical signs associated with visual impairment and behavioural change became obvious at 9 months. Advanced disease reflects the loss of vision, coincided with atrophy of the occipital cortex and loss of photoreceptors in the retina at 18 months. The brain is severely atrophied and little neurons remained at 24 months.

Aberrant pro- and anti-inflammatory cytokine expression is often related to the development of neuroinflammation and progression of disease (Benveniste, 1998). Glial cells are the major immune regulatory cells in the CNS, and they most likely stimulate neurons and other glial cells or infiltrate lymphocytes to produce cytokines (Szelényi, 2001). Cytokines could be released as “danger” signals that trigger an immune response (Allan & Rothwell, 2001). The study of expression of cytokine profiles could provide an insight into how these molecular cues stimulate and/or sustain an immune response in the CLN6 brain. A previous study demonstrated an early elevation of pro-and anti-inflammatory cytokine expression at 6 months, at the initiation of neuroinflammation (Barry, 2011). When these data for all four cytokines are supplemented by 2 month expressions, it became apparent that the expression of cytokines correlates with the initiation of neurodegeneration at 4-6 months of age. The expression however does not correlate with the initiation of glial activation, which began earlier, indicating that the cytokine expression is a consequential event (Fig 4.2).

Other inflammatory studies have also reported that the cytokine expression followed glial activation. TGF-β is expressed in activated astrocytes, microglia and oligodendrocytes and can induce its own gene in an autocrine manner in these cells (Benveniste, 1998; Szelényi, 2001; Spittau et al., 2013). Most studies described the induction of TGF-β expression or its receptors upon glial activation, suggesting TGF-β expression is a consequential event of glial
activation (Mitchell et al., 2014; Pál et al., 2014; Sugimoto et al., 2014). Also, neurons and microglia were reported to produce IL-10 upon glial activation (Szelényi, 2001).

There is a possibility of early activation of cytokines prior to glial activation in the absence of changes in transcription. Cytokine activation can arise from post-translational modification of existent cytokines rather than increased transcription. Pre-existing pro-cytokines may be sufficient for the production of active signals at the early ages of the disease. Both TNF-α and IL-1β have pro-forms, that remain cytoplasmic until they are cleaved enzymatically by the TNF-α converting enzyme matrix metalloprotease and matrix metalloproteinase respectively (Martinon & Tschopp, 2004; Amantea et al., 2007; McCoy & Tansey, 2008). The proteolytic enzymes of the pro-TNF-α and pro-IL-1β were reported to be responsible for the secretion of pro-inflammatory cytokines upon inflammation (Amantea et al., 2007; Lee et al., 2014). In a study of juvenile NCL/CLN3, the primary microglia isolated from CLN3Δex7/8 mutated mice showed an intrinsic hyper-response to the pro-inflammatory cytokines as well as to the inflammatory stimuli released from the JNCL brain cells (Xiong & Kielian, 2013). The hyper-response involved the “inflammasome”-caspase 1 activation which led to subsequent secretion of IL-1β (Xiong & Kielian, 2013).

The roles of the upregulation of these cytokines up-regulation are not very clear. IL-10 receptors have been expressed in a variety of cells in murine and human CNS in basal conditions and after injury. Cells with receptors include neurons, microglia, astrocytes and oligodendrocytes (Ledeboer et al., 2002; Cannella & Raine, 2004; González et al., 2009; Lim et al., 2013; Norden et al., 2014), suggesting that IL-10 may be capable of acting on a broad range of cells. IL-10 and TGF-β are proposed to be anti-inflammatory cytokines but they did not seem to suppress the neuroinflammation in spite of increased expression in the CLN6 brain (Fig 4.2). One explanation is that any anti-inflammatory action of IL-10 and TGF-β in the affected brains may have been limited because of the dramatic intensity of other inflammatory responses (Barry, 2011). TNF-α and IL-1β expressions have been suggested to be the fast response cytokines prior to neuronal death in rodents (Buttini et al., 1994; Liu et al., 1994; Wang et al., 1994). Clinical studies have shown correlation between the concentrations of inflammatory cytokines and the severity of the disease (Griffin et al., 1994; Krupinski et al., 1996; Doty et al., 2015). However, it is difficult to speculate the role of these cytokines in neuronal damage in CLN6 brains since the signalling network of cytokines is complex and the cellular locations of cytokine expression are not determined in this study.
Despite the reports of the cellular location of cytokines, one possibility is that mainly in glial cells, the expression of cytokines does not follow the regional pattern of glial activation (Fig 4.2). In fact, no regional differences were observed in the gene expressions investigated in the present study (Fig 4.2, 4.3, 4.4, 4.5, 4.6 and 4.8). Previous studies had demonstrated the selective survival of the GABAergic interneuron under the influence of glial activation while the gonadotrophin-releasing hormone secreting neurons degenerated in the absence of glial activation, suggesting that the functionality and connectivity of the cells are better determinants of neuronal survival than cell phenotypes (Oswald et al., 2006; Kay et al., 2011). Subpopulations of neurons in some brain regions may be more sensitive to uncontrolled inflammatory signalling than others and other determining factors such as temporal changes in the molecular cues may add to the neuronal stress.

4.5.2 NF-κB 1

The time course of the ubiquitous form of NF-κB 1 gene expression showed its up-regulation is likely to be associated with elevation of cytokine expression, serving a role in amplifying neuroinflammation at the end stage of disease, but follows rather than leads to cytokine expression.

Although the change of NF-κB 1 expression only became apparent at 18 and 24 months (4.8 and 4.1 folds, respectively, Fig 4.3), the amplitude of increase was high. Up-regulation of NF-κB 1 corresponded with peak elevation of TNF-α expression at age of 18 months, which is known to induce NF-κB in murine macrophages (Collart et al., 1990) and human macrophages (Hohmann et al., 1990). Though NF-κB 1 expression declined at 24 months, a 4 fold increase was sustained. IL-1β expression is also reported to activate NF-κB to induce a positive autoregulatory loop in some human cell lines (Hiscott et al., 1993). IL-1β was increased 7.2 fold at 24 month of age (Barry, 2011). NF-κB 1 did not appear to associate with the increase, therefore, IL-1β may not be the primary target of NF-κB.

The ability of NF-κB to respond to a signal does not require transcriptional activation (Ghosh et al., 1998) and the possibility of an early activation of NF-κB cannot be excluded. The subunit p50 is a post-translational product of a larger product (p105) which encoded by the gene NF-κB 1. The p105 is exclusively cytosolic, attributed to masking of the nuclear localisation signals (NLS) by the C-terminal ankyrin-repeat region (Henkel et al., 1992). This indicates another possible level of control of NF-κB activation. Pre-existing p105 could be
processed rapidly upon stimulation and activated without active transcription. Therefore, NF-κB could be activated without more being translated. Moreover, the duration of NF-κB activation is not known since it is correlated with both the synthesis and degradation of NF-κB inhibitors (IκB proteins). IκB binds to heterodimers (i.e. p50/p65), thereby masking its nuclear localization sequence and inhibiting its DNA-binding activity (Ghosh et al, 1998). The differential degradation rates of free and bound IκB proteins were proposed to have an impact on constitutive NF-κB activity while the NF-κB activity also plays a role in transcriptional regulation of these inhibitors to control its own steady-state activity (O’Dea et al, 2007).

4.5.3 MAPK14

Expression of the p38α gene, MAPK14, only showed significant increase in the advanced disease, indicating MAPK14 expression is not affected in disease progression (Fig 4.4). However, this may not fully represent the activation of the p38α MAPK signalling pathway in the disease progression, since again activation of such pathway greatly relies on post-translational modification. p38α MAPK is activated when threonine and tyrosine in the activation loop are phosphorylated (Huang et al, 2009). The phosphorylated substrate of p38α MAPK or phosphorylated forms of p38α MAPK are markers for p38α MAPK activation (Lo et al, 2014). It is also reasonable to consider the impact of the down-regulatory mechanism by protein phosphatases that may also play a role in the dynamic regulation of MAPK14 expression (Hargrove & Schmidt, 1989).

The brain specific isoform p38β may be preferentially activated in the CLN6 affected brain, though it is potentially functionally redundant in the presence of p38α (Section 4.3.3). Nevertheless, p38β demonstrated substrate preference in vitro. For instance, activating transcriptional factor 2 (ATF2) was shown to be better phosphorylated by p38β, indicating a different regulatory mechanism of p38β on the downstream targets (Jiang et al, 1996). Therefore, p38β may be selectively activated to act on specific substrates.

4.5.4 JAK2/STAT1/STAT3

Similarly to the other transcriptional factors, JAK2/STAT1/STAT3 were not up-regulated at early stage of the disease progression (Fig 4.5). This indicates that the pathway is not likely causative. The change of JAK2 expression was barely 2 fold at terminal disease. STAT1 and STAT3 expression increased in the affected animals at 9 months, and peaked at 18 months.
Expression of all these genes decreased but remained higher than the controls at 24 months. The high level of variation indicate evident differences in expression between biological replicates and leads to doubts that the apparent up-regulation of these genes are truly biologically important in the pathogenesis.

There were high levels of variation between biological replicates in JAK2 expression in both genotypes and in the affected animals for STAT1 and STAT3. Large differences between PCR replicates and biological replicates could occur in quantitative real-time PCR, due to the chemistry of PCR reaction when genes are expressed in low copy numbers (<1000 starting copy) (Peccoud & Jacob, 1996; Karrer et al, 1995). This, in theory, could partially result from the reduced collision frequency of primers and templates thereby reduced chances of contact between the primers and the respective templates (Ruano et al, 1991). JAK2, STAT1 and STAT3 had starting copies that are more than 1000 and the variation only represent the differences between biological replicates, not PCR replicates. Thus, technical limitations do not account for the large variation.

It is not surprising that the affected animals showed slightly higher expression since the product of the mutated CLN6 may cause a general metabolic disturbance in cells and affect transcriptional expression. This should affect all the affected animals equally. Therefore, the differences of expression between the biological replicates may not be disease-related and may arise from the responses of the individuals.

In addition, the variable expression between biological replicates could well be a sampling issue. The expression of these genes could be variable in cell types and brain regions and this has been demonstrated in Chapter 3.

Activation of JAK/STAT is likewise post-translational, by phosphorylation of JAK2 tandem tyrosine residues in an activation loop and STAT1 and STAT3 on serines and tyrosines (Hornbeck et al, 2012). Again like NF-κB, the JAK/STAT pathway can be activated without active transcription.
4.5.5 SOCS3

4.5.5.1 SOCS3 expression is associated with an irregular immune response

The up-regulation of SOCS3 (Fig 4.6; Table 4.5) in the CLN6 brains are not understood since the primary up-stream regulators of SOCS3, IL-10 (Fig 4.2) and STAT3 (Fig 4.5) were not up-regulated prior to its up-regulation at 2 months. The role of SOCS3 is also ambiguous in relation to its possible function in the CLN6 brain.

The early response of SOCS expression may indicate an inhibition of glial activation (Fig 4.6). Immunoregulatory roles of SOCS3 have been proposed to suppress activation of immune cells in mice (Lee et al., 2002; Croker et al., 2003; Panopoulos et al., 2006) and human (Hörtner et al., 2002) at an developmental stage for cell differentiation such as haematopoiesis (Panopoulos et al., 2006; O’Shea & Murray, 2008). G-CSF, a cytokine, in part stimulates maturation and exit of neutrophils from bone marrow (Eyles et al., 2006) via its receptor G-CSFR, activates STAT3 and triggers SOCS3 expression (Lee et al., 2002; Hörtner et al., 2002; Croker et al., 2003). The SOCS3 may then down-regulates the signalling pathway via inhibiting G-CSFR and STAT3, resulting in a reduction of neutrophils (Lee et al., 2002; Hörtner et al., 2002).

SOCS3 expression was proposed to have different effects depending on the cell types in mice. SOCS3 expression in glial cells appears to have an anti-inflammatory role. In astrocytes, IFN-β induces the expression of SOCS3 in a STAT3 dependent manner (Qin et al., 2008). Disruption of SOCS3 production leads to enhanced production of chemokines that promote migration of microglia and T cells (Qin et al., 2008). The mechanism of SOCS3 mediated anti-inflammation has been studied. IL-10/IL10R/STAT3 induced SOCS3 expression competes with the STAT3 binding site on the IL-6 receptor (gp130), thus preventing the recruitment of the down-stream signalling complex (Section 4.3.4). IL-10 induced SOCS3 expression was reported to down-regulate inflammatory genes, partially via inhibition of IL-6 receptor binding (Qin et al., 2006). On the other hand, SOCS over-expression in neurons have been suggested to have a deleterious effect on neuronal survival. Evidence suggests that over-expression of SOCS3 reduces neural survival and deletion of SOCS3 promotes neural repair (Miao et al., 2006; Smith et al., 2009a; Sun et al., 2011; Park et al., 2014).

SOCS3 appears to have both pro- and anti-inflammatory actions often associated with STAT3 activation. However, STAT3 was not up-regulated prior to SOCS3 activation in the early
disease (Fig 4.5; 4.6), suggesting either a transient activation of STAT3 or STAT3-independent activation. SOCS3 is known to primarily and negatively regulate JAK/STAT1/3 to restore the balance of the molecular environment. Supposedly, a positive feedback triggered the SOCS3 expression and it is unlikely to be the JAK/STAT pathway, because that pathway did not seem to be activated as dramatically as SOCS3 (Table 4.4; Fig 4.5). Studies have found SOCS3 can be induced by growth factors, growth hormone, leptin and insulin (Bjørbaek et al, 1998 in vivo, mice; Cacalano et al, 2001 in vitro, humans; Peraldi et al, 2001 in vitro, mice), suggesting SOCS3 expression can be induced via means other than the classical JAK/STAT pathway.

A mutation in CLN6 may have an effect on the regulation of SOCS3 expression, but without knowing the exact function of CLN6, it is difficult to rationalise the correlation. IL-10 is proposed to be implicated in up-stream SOCS3 signalling (Qin et al, 2008), but did not show parallel up-regulation in early disease progression, adding to the difficulty of interpreting the SOCS3 elevation.

4.5.5.2 SOCS3 expression and microglial phenotype

SOCS3 has been proposed to be a marker for polarised microglia in response to stress (Chhor et al, 2013). Activated microglia are thought to be able to adopt distinct phenotypes and switch between phenotypes, depending on the disease type and the progression (Perry et al, 2010). Their expression of the cell surface receptors and release of soluble factors in response to chemical or cell specific cues are proposed to distinguish the different phenotypes (Perry et al, 2010; Chhor et al, 2013). Similarly to macrophages, microglial phenotypes can be classified as M1 (cytotoxic) and M2 (immunoregulatory). M2 is further divided into M2a (repair and regeneration), M2b (immunoregulatory) and M2c (acquired – deactivating) (Chhor et al, 2013). However, the morphology of microglia do not reflect changes of their phenotype (Perry et al, 2010). The surface receptors and secretion of molecules differs between phenotypes. SOCS3 was recognised to be up-regulated with IL-1R, a known marker for M2b, and was proposed to be a novel marker for M2b (Chhor et al, 2013). The early response of SOCS3 in the CLN6 affected brain may due to a change of microglial phenotype. The longitudinal study on glial activation previously used MHC II and GSB4 to label activated microglia (Oswald et al, 2005). There is a lack of information as to whether MHC II and GSB4 are able to differentiate phenotypic changes of microglia.
Nonetheless, they detected activated microglia as early as 12 days in the CLN6 affected parieto-occipital cortex (Oswald et al., 2005). This microglial activation is region-specific and is not correlated with a general up-regulation of SOCS3 in the cortex (Fig 4.6).

4.5.5.3 SOCS3 protein detection

Expression of SOCS3 protein was investigated by Western blotting (Fig 4.7). The protein expression increased dramatically in the affected animals at the ages of 9 and 18 months, correlating with the elevation of gene expression (Fig 4.7). This was not observed at earlier ages, perhaps because the low abundance and short protein half-life made it difficult to be detected.

There is little expression of SOCS3 protein in the control sheep. It was difficult to detect any signals when 25µg of soluble protein was loaded. Doubling amount of loading and longer exposure of films were required to produce visible signals. Studies have found features of SOCS3 in cells that either interact with elongin B/C, or bear phosphorylated tyrosine residues Tyr204 and Tyr221 (Zhang et al., 1999; Haan et al., 2003). The latter form has been shown to enhance proteasome-mediated degradation of SOCS3 (Haan et al., 2003). It has also been suggested to support cytokine and growth factor signalling, such as by IL-2, EPO, EGF and PDGF (Cacalano et al., 2001). In addition, SOCS3 has a PEST motif in its SH2 domain which appears to mediate non-proteasomal degradation (Babon et al., 2006). In spite of a positive feedback role of the phosphorylated SOCS3 in signal transduction, post-translational modifications seem to aim for a fast degradation of SOCS3, suggesting a high turn-over of the protein. The short half-life of SOCS3 may cause difficulties for detection for the protein.

SOCS3 mRNA expression is significantly higher at the ages of 2 and 6 months in the affected animals, however copy numbers are not high, suggesting that the protein concentrations may still not be sufficient for detection at those ages.

4.5.5.4 SOCS3 mRNA and protein correlation

Immunoreactive SOCS3 was only visible in the affected animals at 9 and 18 months, confirming the disease-related role of SOCS3 (Fig 4.7). That the affected frontal cortex did not show immunoreactivity at 9 months is likely because the amount of protein was not enough for detection (Fig 4.7). The significant fold change for SOCS3 was 21 and 74 at 9 and 18 months respectively (Table 4.5). The intensity of the immunoreactive band appeared to
be much stronger at 18 months than at 9 months (Fig 4.7), indicating a parallel increase in protein expression. However the change of protein expression appeared to be more dramatic than the change of transcription between 9 and 18 months (Table 4.5; Fig 4.7). Correlations between mRNA transcription and protein translation as well as protein degradation could be subject to variable degrees of disruption when disease progresses. The transcriptional and translational machinery and protein degradation in cells may be less tightly controlled due to the severity of the disease. Observations of a widespread up-regulation of all the genes towards the end stage of the disease at 18 months indicates an uncontrolled response. Presumably the correlation between mRNA and protein would become more discordant as disease progresses. Other possibilities could be involved, such as the samples were from the degenerative region that had higher concentration of the protein (Section 3.4.2; 3.4.3) and individual differences in protein expression between sheep.

4.5.6 BDNF and Trk B

BDNF did not appear to associate with disease progression but the affected animals do appear to have a large difference in expression at 2 months (Fig 4.8A). This difference was caused by one animal showing much higher expression. The same animal did not produce variable expressions for the other genes investigated. It is not clear what caused the variation of BDNF expression in this particular affected animal. Stimuli such as deprivation of light (which causes reduction of mRNA), electroconvulsive seizure, mechanical stimulation and physical exercise can physiologically alter BDNF expression (Castrén et al, 1992; Neeper et al, 1995; Rocamora et al, 1996; Dias et al, 2003).

The difference in copy numbers and expression pattern were found between BDNF and Trk B. Differences are most likely attributable to the difference in localisation between these molecules. Expression of BDNF and Trk B did not appear to correlate precisely in cells and brain regions in adult mice and human (Murer et al, 1999; Tang et al, 2010; Marco-Salazar et al, 2014).

The overall objective of this study was to probe whether intracellular vesicle sorting of BDNF and Trk B was interrupted by mutated CLN6. The relatively stable expression of BDNF in affected brains suggests that BDNF sorting may not be disrupted. The Trk B expression pattern in the affected animals resembled that of most of the genes investigated in that it
peaked at 18 months and declined in 24 months (Fig 4.8B), emphasising the possibility that there is a global metabolic dysfunction of cells at terminal disease.

4.5.7 Sampling error

The observation of immunostaining for mitochondrial proteins revealed a sampling problem associated with the uneven distribution of their expression (Section 3.4.2; 3.4.3). It should be recognised that the selected neuroinflammatory genes could be subject to the same sampling problem, since the same samples were used for the detection of mitochondrial genes. As all these genes showed a higher expression at late disease stages, it may mean there are changes in anatomical and cytoarchitectural structure in the CLN6 brains rather than an intrinsic cellular change in activities, except for SOCS3 which increased its expression when the brain remained intact at early developmental stages. However, SOCS3 protein and gene expression could be affected by sampling error in later disease progression.

4.5.8 Activation of signalling pathway

The uncertainty in the current study as to whether mRNA levels are reflective of protein expression and activation of the signalling pathway is difficult to answer. Statistical studies on the collection of genes and their corresponding proteins showed mRNA-protein correlation strength could vary between genes, indicating that mRNA levels are not necessarily reflective of protein expression (Östlund & Sonnhammer, 2012; Guo et al, 2008).

Theoretical mathematical models for mRNA and protein induction is able to predict:

“The concentration of protein at steady state equals the product of the rate constants for synthesis of mRNA and protein (ks1 and ks2) divided by the product of the rate constants for degradation (kd1, and kd2) and that the rate at which protein concentration changes depend on the rate constants for degradation of both the mRNA and the protein. This permits great flexibility in controlling induction kinetics for particular gene products, since their synthesis, translation, and degradation may be regulated co-ordinately to permit induction to be stable or transient or to amplify the final yield of protein.”

-Hargrove and Schmidt, 1989
The mathematical model was built on the premise that mRNA and protein expression is correlated and the biological importance of such correlation. mRNAs of transcriptional factors are generally short-lived (Wang et al., 2002b; Yang et al., 2002; Sharova et al., 2009), suggesting that mRNA of transcription factors is sensitive to transcriptional regulation under stress conditions (Dey et al., 2015). Therefore, studies of mRNA levels of the transcription factors as a predictor of their functional protein expression may not be entirely invalid. Although the translational modification is significant in the activation of proteins, transcriptional regulation also plays a role in maintaining the steady-state level of a protein (Hargrove & Schmidt, 1989). Depletion of non-phosphorylated forms of a molecule in cytosolic pools is likely to trigger transcription of the molecules to sustain an overall level. Measurements of phosphorylated forms of the signalling molecules would be informative as to whether the signalling pathway is temporarily activated when the threshold of transcription is not reached. For instance, STAT3 may be temporally phosphorylated to trigger transcription of SOCS3 without changing the number of transcripts.

4.5.9 Lymphocytic infiltration and IgG deposition

Evidence of a BBB breach has been found in some murine NCL models and in one human case (Lim et al., 2007; Saha et al., 2012; Groh et al., 2013). However, a lack of immunostaining for lymphocytic markers in the CLN6 affected brain, and the lack of deposition of immunoglobulin (IgG) beyond the intravascular space, indicates that the BBB integrity is not disrupted in the ovine CLN6 affected brain (Fig 4.11).

IgG staining revealed uptake of IgG into perivascular space, neurons, and occasional astrocytes in the entorhinal cortex and the hippocampus in human JNCL. CLN3\(-/-\) mice also showed neuronal uptake of IgG in the cortex and hippocampus. In controls of both experiments, IgG deposition was reported within the blood vessels (Lim et al., 2007). This observation could possibly arise from sample handling. An early investigation demonstrated that extravascular leakage leading to cellular uptake is dependent of the time between death and autopsy (Mori et al., 1991). Serum protein leakage could evolve from focal to diffuse patterns and also caused by mishandling of post mortem tissue (Mori et al., 1991). In the ovine brain, IgG deposition was only found in intravascular space regardless of genotypes, brain regions, and ages. The staining was evidently not an exclusive characteristic of the affected animals, indicating coagulation of the residual blood post mortem due to capillary action caused by the small size of the blood vessels in the brain.
Moreover, other data in Lim et al (2007) cannot adequately support the hypothesis of the BBB breach in the disease mechanism. Deposition of immunoglobulins, and infiltration of lymphocytes in the CNS was found in human JNCL autopsy material at terminal stage of the disease, thus does not indicate a pathogenic role of the BBB breach in disease progression. In the murine JNCL (CLN3−/−), the immunohistochemical and western blotting analyses of tight junction proteins, which facilitate a barrier function by maintaining tight junction integrity, did not reveal changes of expression (Lim et al, 2007).

In another report regarding the BBB disruption of human INCL (PPT1), increased expression of matrix metalloproteinases (MMPs) found in one affected human brain, was thought to be potentially activated by cytokine released from the infiltrated lymphocytes (Saha et al, 2012). This correlation was suggested based on a murine study (Saha et al, 2012). T<sub>H</sub>17 cells were found in the brain of Ppt-KO mice (INCL). In cell cultures, MMPs were increased in murine brain endothelial cells treated with IL-17A, which could normally be produced by T<sub>H</sub>17 cells. The assumption of this correlation is challenged by the lack of indication of infiltration of T<sub>H</sub>17 cells and of perturbation of the BBB in the INCL human brain (Saha et al, 2012).

Breaches of the blood-brain-barrier have been considered as a possible underlying mechanism for neurodegenerative diseases. Despite the fact that small animal models could be informative in understanding the mechanism of neurodegeneration, they do not completely recapitulate human disease, especially as the human brain has a more complicated anatomy and physiology. Research scientists often work in a reductionist way. For instance, human AD displays both amyloidopathy and tauopathy while small animal models were developed to focus on one or the other (Institute of Medicine, 2013). Studies of these animals are subjected to the constraint of their short life span, whereas the human disease occurs and evolves over years. Animal models with knock-out genes are not likely to replicate human disease, much less so when the diseases are caused by genetic mutations that may enable the production of protein that may retain partial or altered function that play a part in the pathogenesis.

An adaptive immune response is considered to be a secondary response following the innate response initiated by glial activation. The development and severity of the secondary response may vary between species. For instance, SOD1 mice showed permeability changes
and microhaemorrhages that implied severe vascular damage in murine ALS while microbleeds were not observed in human ALS, regardless of regional hypoperfusion (Evans et al., 2013). Although there is a lack of preliminary data relating to the BBB disruption in human NCL, an alteration of the BBB cannot be completely precluded. The end-stage characteristics of cerebellar pathology of human CLN6 may not be seen in the affected sheep as the affected sheep may not live long enough (Oswald et al., 2005). Similarly, the BBB breach may be part of the pathogenic mechanism of human disease but the ovine model does not exhibit the BBB breach because the animals were sacrificed before natural death for humane reasons. Current clinical practice involving BBB imaging makes it possible to measure the BBB permeability (Veksler et al., 2014). A way forward is that non-invasive assessment of the human BBB integrity by MRI, which is able to directly investigate human cases in a timely manner and in large numbers, to characterise if a BBB disruption is present and consistent among NCL patients.

4.6 Conclusion

The study revealed that only SOCS3 changes are meaningful to the neuropathogenesis while changes in the other selected genes are possibly contributing factors, but do not directly cause the disease.

It was not expected that the majority of the selected neuroinflammatory genes shared similar expression patterns in which expression increased between neurodegeneration initiation (6 months) to terminal disease (18 months) then declined in the severely atrophied and dying brain (24 months). The exception was SOCS3 expression, which was elevated as early as 2 months of age in parallel with the initiation of neuroinflammation. This suggests a general metabolic disruption of cells following glial activation rather than being causative. The cell types involved are not clear. Up-regulation of the genes could be inflated or underestimated depending on shifts in cell populations concomitant with the loss of neurons and the increase of glial cells. Measurements of mRNA in specific cell populations can be a future direction as could use cell-specific markers in conjunction with the endogenous references genes.

Regional differences in gene expression were not found. Physiological functionality and interconnectivity determines the cell fate (Oswald et al., 2006, Kay et al., 2011). The contradictory roles of SOCS3 suggest the same molecule may have different actions
depending on the physiological functions of the cells. The intercellular signalling may be subtle, temporal and hard to be detected using the current post-mortem investigation.

The SOCS3 elevation at 2 months is probably a genuine response as the cytoarchitecture remains intact. It is likely associated with a primary response. It is not understood what triggered the up-regulation. STAT3 is known to transcribe the SOCS3 gene, and may be temporally activated without a change of mRNA expression. However, the underlying mechanism of this elevation requires further investigation.

There was no evidence of BBB disruption in the ovine CLN6 brain, suggesting the ovine model at 24 months does not reflect the terminal human pathology. However, the BBB disruption has not been proven to be a general pathology in human NCL. If the BBB disruption is part of the terminal pathology in human NCLs, any adaptive immune response that occurs at the terminal stage of the human disease may not be additive to the neuroinflammation but a sign of virtually dead brain.
Chapter 5
Investigation of metallothioneins, progranulins and the PI3K pathway in ovine CLN6 brain

5.1 Introduction

Investigations of the neuroinflammatory cascade revealed an unanticipated generalised response of neuroinflammatory mediators that followed glial activation. This response chronically increased, then subsided in the dying brain. The exception was a significant increase of SOCS3 expression at the initiation of glial activation which progressively increased throughout the disease progression. However, the role of the SOCS3 up-regulation is not understood since no elevation of its upstream modulators was detected. Evidence indicate a complex signalling network in the disease. Factors contributing to the signalling network complexity may include diverse cell types, the cellular locations of the neuroinflammatory modulators and the interconnectivity of brain regions.

Other genes may be involved in the neuroinflammatory mechanisms of NCLs. Extensive signalling pathways could be interwoven to be suppressive, additive or synergistic. Changes in the balance of the signalling may lead to a pro-inflammatory environment, which is damaging to the vulnerable neurons. Exploring other possible lesions at early stages of the disease progression may lead to a better understanding of the disease mechanisms.

5.1.1 Metallothioneins (MTs)

5.1.1.1 MTs, neuroinflammation and lysosomal storage diseases (LSDs)

Up-regulation of metallothioneins (MTs) has been found in a number of neurodegenerative diseases, including AD, ALS and MS, as well as in brain injuries that were associated with neuroinflammation and oxidative stress (Ebadi et al, 1995). Significant up-regulation of the MT oligomers and polymers was notable and consistent at 12-14 months in the CLN6 affected animals, the polymers being more significantly expressed (Kanninen et al, 2013). Although the MT antibody used was not specified in the Kanninen study, the MTs detected were likely to be MT I or II. Moreover, a recent study reported over-expression of MTs in the brains of patients with a range of lysosomal storage diseases that included NCL (Cesani et al, 2014), however the CLN mutation was however not specified. The underlying cause of such up-regulation remains obscure.
MT expression may play a role in neuroinflammation but it is not clear at which point in the neuroinflammatory pathway. The consistent expression of MTs in astrocytes and their association with inflammatory mediators suggest they may play a role in the regulation of inflammation. These inflammatory mediators include glucocorticoid, IL-6 and the TNF-α type 1 receptors (Ebadi et al, 1995; Carrasco et al, 1998; Carrasco et al, 2006; Lee et al, 2010; Martinho et al, 2013; Cesani et al, 2014; Chen et al, 2014).

5.1.1.2 MTs as metal chelators

MTs have a high content of cysteine residues through which they are capable of binding 4-12 heavy metal atoms (Ebadi et al, 1995). Four types of MTs have been discovered, MT I and II are ubiquitously expressed, MT III is brain specific and MT IV is restricted to some stratified squamous epithelia. The metal binding ability suggests that MTs may be associated with the regulation of the cellular metal homeostasis and heavy metal detoxification (Santos et al, 2012).

Chelatable Zn$^{2+}$ participates in synaptic transmission, modulation of membrane receptors/channels and the regulation of intracellular Ca$^{2+}$ (Sekler & Siverman, 2012). Changes of Zn$^{2+}$ concentrations were shown to regulate MT expression in the brain and a role for MTs has been suggested to buffer cytosolic Zn$^{2+}$ released by neurons (Ebadi et al, 1995, Sensi et al, 2009). MTs were found to be taken up by cells through lipid raft-dependent endocytosis while the apohioneins (MTs without bound metal) remain in the intracellular vesicles. The metals bound to MTs were released into the cytosol, suggesting a pathway for cellular acquisition for metals (Hao et al, 2007).

5.1.2 Progranulin

5.1.2.1 Progranulin and neuroinflammation

Progranulin (PGRN, encoded by GRN) is distributed widely throughout the body, including inneurons and activated microglia in the CNS (Ryan et al, 2009; Ahmed et al, 2010; Petkau et al, 2010; Petkau & Leavitt, 2014). It was found to bind sortilin or prosaposin, which facilitate PGRN transport to lysosomes (Hu et al, 2010, Zhou et al, 2015). Another study by Capell et al (2011) disagreed with the lysosomal localisation of PGRN. This study instead found that PGRN localises mainly within the ER and the Golgi network. Treatment with lysosomal protease inhibitors had no effect on PGRN expression and distribution, or its accumulation in lysosomes. Moreover, BafA1, a vacuolar-ATPase (v-ATPase) inhibitor, which leads to
impaired lysosomal degradation, successfully increased PGRN in the ER and Golgi network but not in the lysosome or autophagosome.

Progranulin is also a secretory glycoprotein which can be detected in the blood and the CSF (Zhou et al., 1993; Van Damme et al., 2008). It is proteolytically cleaved by neutrophil proteases to produce smaller proteins, namely granulins (Petkau & Leavitt, 2014). The exact function of progranulin in the brain is unknown. Progranulin was consistently reported to be up-regulated in astrocytomas and in activated microglia, suggesting a role in neuroinflammation (Petkau & Leavitt, 2014).

5.1.2.2 Progranulin and FTLD

Progranulin attracted attention when it was discovered that heterozygous mutations in its gene, GRN, cause fronto-temporal lobar dementia (FTLD). The most common FTLD, FTLD-TDP43, is characterised by pathological inclusions of a hyper-phosphorylated and ubiquitinated trans-activating DNA binding protein with a molecular weight of 43kDa (TDP-43), and ubiquitin in cells in the cortex, hippocampus and corpus striatum (Cairns et al., 2007). In general, the affected patients show symmetrical focal atrophy of the frontal or temporal lobes, or both, as well as occasional asymmetrical atrophy (Cairns et al., 2007). Clinical symptoms include frontotemporal dementia, dysfunction in semantic language (termed semantic dementia, SD), and speech impairment (termed primary progressive non-Xuent aphasia, PNFA). Overlapping clinical phenotypes relate to neuropathologic lesions including motor neuron disease (MND)/amyotrophic lateral sclerosis (ALS) and Parkinsonism (Cairns et al., 2007). Heterozygous mutations in GRN result in losses-of-function that lead to a 30% protein reduction and a 50% mRNA loss, suggesting a haplo-insufficient nature (Petkau & Leavitt, 2014).

5.1.2.3 Progranulin and NCLs

A recent report proposed that homozygous mutations of the progranulin gene (GRN) result in a new form of NCL, based on the resemblance of fingerprint inclusions and some phenotypic clinical symptoms observed in patients (Smith et al., 2012). NCL inclusions of similar morphology were found in GRN/- mice (Ahmed et al., 2010; Petkau et al., 2012; Smith et al., 2012). In addition to other findings in regard to progranulin, it has been suggested that progranulin may be associated with lysosomal function (Götzl et al., 2014). Firstly, progranulin was found to be endocytosed by neurons via the binding of sortilin, which is a transport receptor involved in the targeting of soluble lysosomal proteins (Braulke &
Bonifacino, 2009; Hu et al, 2010). Secondly, a strong risk factor, TMEM106B, found to associate with endosomes and lysosomes, was suggested to play a role in the pathogenic mechanisms related to progranulin haplo-insufficiency (Van Deerlin et al, 2010; Cruchaga et al, 2011; Finch et al, 2011; Nicholson et al, 2013). Recently, TMEM106B has been shown to be a glycoprotein, predominantly localised on the lysosomal membrane and possibly late endosomes, where it may interact with progranulin thus affecting its processing and consequently leading to a reduction of granulin (Brady et al, 2012; Chen-Plotkin et al, 2012; Lang et al, 2012; Nicholson et al, 2013). Lastly, a study that probed the proteins stored in animal models and humans with NCLs and FTLD, showed similar protein storage profiles between the two diseases (Götzl et al, 2014). This indicated a common pathobiochemistry in the two neurodegenerative diseases and suggested that they shared a pathogenic mechanism, possibly through mediation of TMEM106B and progranulin (Götzl et al, 2014). Investigations of progranulin expression in the well-established ovine CLN6 and healthy counterparts may provide insights into the correlation of NCLs with progranulin and further the understanding of the basic neuroinflammatory cascade.

5.1.3 PI3K pathway

Studies of phosphatidylinositol-3 kinase (PI3K) can be important for exploring the regulation of neuroinflammation as well as detection of disruptions in the endoplasmic reticulum–Golgi–endosome–lysosome (GERL) pathway. PI3K is a group of enzymes that phosphorylate the 3-position of the inositol ring of phosphoinositides to produce phosphatidylinositol (3) monophosphate (PIP3), phosphatidylinositol (3,4) bisphosphate (PI(3,4)P2), and phosphatidylinositol (3,4,5) triphosphate (PI(3,4,5)P3). These in turn activate the downstream signalling pathways mammalian target of rapamycin (mTOR) or MAPK, leading to cell growth and survival, cytoskeletal remodelling, immune responses (cytokine production and autophagy), synaptic plasticity and the trafficking of intracellular vesicles (Koyasu, 2003; Li et al, 2010; Knafo & Esteban, 2012; So & Fruman, 2012). Among the four classes of PI3Ks (Iα, Iβ, II and III), PI3K IA and PI3K III are those of interest for this study because of their roles in the regulation of inflammation and mediation of vesicle transport.

5.1.3.1 PI3K I and neuroinflammation

PI3K IA enzymes are heterodimers composed of an 85/55/50 kDa regulatory/adapter subunit (p85) and a 110kDa catalytic subunit (p110). The class I enzymes use PI, PI(4)P, or PI(4,5)P2 as substrates to generate PI(3,4,5)P3 in vivo (Koyasu, 2003; Backer, 2010). Class IA PI3Ks can
be activated by tyrosine-kinase-associated receptors, including toll-like receptors (TLRs) and cytokine receptors in immune cells such as T cells and dendritic cells (DCs). Upon activation by cytokines, the phosphotyrosine motifs (pYxxM) in the receptor tyrosine kinases recruit PI3K which induces conformational changes that activate the catalytic subunit. Activated PI3K then catalyses phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3,4,5)-triphosphate (PIP3). Downstream PIP3 activates kinases, such as PDK1 and AKT/PKB to act on multiple cellular pathways.

Neuroinflammation is marked by elevated cytokines and related signalling molecules, as shown in the previous chapter of this study. The immuno-regulatory function of the PI3K signalling pathway is implicated in the maintenance of proper adaptive immunity, self-tolerance in lymphocytes and activation of autophagy (So & Fruman, 2012). Activation of the PI3K IA- Akt signalling pathway is thought to be pro-survival and anti-apoptotic via inhibition of apoptotic molecules such as GSK3β, Bcl-2 associated death protein (BAD), and forkhead transcription factor (FKHR) (Datta et al, 1997; Franke et al, 2003). Activation of Akt has also been shown to inhibit the formation of autophagosomes by phosphorylating downstream targets, including the mammalian target of rapamycin (mTOR). Regionally specific activation of PI3K IA in the parietal and occipital cortex was reported in the CLN6 ovine brain and was suggested to relate to the PI3K IA-GSK3β pathway (Kanninen et al, 2013), hence the activation of PI3K signalling may be associated with the mediation of cytokine signalling in neuroinflammation in the NCLs. Cytokine signalling, such as by IL-1, is mediated by activation of a receptor complex (IL-1R1, IL-1RAcP) and an adaptor molecule (MyD88), which lies upstream of PI3K (Viviani et al, 2004; Li et al, 2010). Meanwhile, IL-10 was also reported to be up-regulated by the PI3K signalling pathway (Tapia-Abellán et al, 2012).

### 5.1.3.2 PI3K, autophagy and endocytic pathway

There have been numerous indications of disturbances in the endosome–lysosome pathway and suggestions of problems with autophagy in NCLs. PI3K III is thought to mediate autophagy by forming a protein complex containing Beclin 1, a novel Bcl-2 interacting autophagic protein that is the mammalian homolog of the ATG6 gene in yeast promoting autophagy (Liang et al, 1999; Kihara et al, 2001). Although PI3K signalling has been reported to be impaired in CLN3 and CLN10 and proposed to be caused by autophagic stress (Cao et al, 2006; Walls et al, 2007; Chang et al, 2011), hyper-active autophagy is not a common feature of the large number of histological and ultrastructural studies of tissues from
patients and animal models (Palmer et al, 2013). Any alteration in PI3K signalling is more likely to be associated with the defective endocytic pathway.

Some NCL proteins have been predicted to be components of GERL pathway, suggesting the pathway may be defective. Disturbed endocytosis and intracellular trafficking has been reported in NCL studies including CLN1, CLN2, CLN3, and CLN5, mostly in cell culture studies (Fossale et al, 2004; Kyttälä et al, 2004; Luiro et al, 2001; 2004; Kopan et al, 2004; Buff et al, 2007; Saja et al, 2010; Mamo et al, 2012; Uusi-Rauva et al, 2012).

There is evidence that showed inhibition of PI3Ks pathway impaired the targeting of procathepsin D from the trans-Golgi network (TGN) to the lysosomal compartment and resulted in intracellular accumulation of unprocessed procathepsin D in yeast and in mammalian cells (Davidson, 1995; Brown et al, 1995). In plant cells, inhibition of PI3Ks led to swelling and aggregation of late endosomes that consequently affected degradation of proteins (Takáč et al, 2012; 2013). In these studies, non-selective inhibition of PI3Ks by wortmannin and Y294002 did not reveal specific functions of different classes of PI3K in GERL pathways.

Constitutive generation of PI(3)P by PI3K III is conserved from lower eukaryotes to plants and mammals, and is acquired as a lipid component of the endosomal compartment (Engelman et al, 2006). PI3K III generates PI(3)P that regulates membrane trafficking processes via recruitment of a subset of proteins containing PI(3)P-binding PX (phox homology) and FYVE domains (Koyasu, 2003). Specific FYVE-domain proteins such as early endosomal antigen 1 (EEA1), interact with Rab5 GTPases that control vesicle docking and fusion in the early endocytic pathway (Falasca & Maffucci, 2009). Therefore, PI3K III may be important to the transport and sorting of proteins in the GERL pathway.

Studies have suggested that PI3K III and I are not redundant in the GERL pathway in cells and may participate in different stages. It was reported that PI3K III is involved in phagosome formation and maturation, whereas PI3K I is required for optimal phagocytosis in human macrophages (Vieira et al, 2001). A reduction of PI3K III expression in human glioblastoma cells did not affect the normal endocytosis and protein trafficking from the TGN, but caused defects in the late endosomal department. These defects were manifested by enlarged endosomes and a reduced rate of processing and sorting of proteins to lysosomes (Johnson et al, 2006). Hypothetically, alteration in PI3K I and III expression may result in disruption of endocytic pathway, thus raising the possibility that there is defective vesicle formation,
causing an improper incorporation of normal subunit c in the endocytic department which in turn leads to its accumulation. The alteration of PI3K I and III expression could be detected by antibodies. The activation of signalling pathway by PI3K I could be detected by probing the amount of phosphorylated Akt, which is a downstream mediator of PI3K I.

5.2 Materials and methods

5.2.1 Animals

As described in Chapter 2.

5.2.2 Tissue collection for mRNA and protein

Brains were collected and processed from the same animals as used for gene expression studies in previous chapters, described in sections 2.2.1 and 2.2.2 for mRNA and protein analyses respectively.

5.2.3 RNA isolation and cDNA synthesis

As described in section 2.3.1.

5.2.4 Primer design and plasmid generation

These were carried out according to the protocol (Chapter 2.3). MgCl₂ concentrations and temperatures were adjusted to improve the stringency of PCRs (Table 5.1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence reference in GenBank</th>
<th>Tₐ*</th>
<th>MgCl₂ (mM)</th>
<th>Primers</th>
<th>Expected product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine MT IA</td>
<td>XM_004014996.1</td>
<td>57 °C</td>
<td>3</td>
<td>External F- GCTTGCCACTTGTTCGAC</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R- GCACCAGCTCAGGGTTGTATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>59 °C</td>
<td>2</td>
<td>Internal F- TGGATCTGCTTGGCCACTTGT</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R- AGCAGCAGCTCTTTCTTGCAG</td>
<td></td>
</tr>
<tr>
<td>Ovine MT IIA</td>
<td>XM_004014999.1</td>
<td>57 °C</td>
<td>3</td>
<td>External F- CCTCGCCATCCTTTGCCCTCAG</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R- GGGATGTAACAAACAGGGGTCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 °C</td>
<td>3</td>
<td>Internal F- CCATCTTTGCTCAGCCGTT R- TCTTTGCATTGTCAGGGGACC</td>
<td>118</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-------</td>
<td>---</td>
<td>----------------------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Ovine MT III</td>
<td>AF500199.1</td>
<td>57 °C</td>
<td>3</td>
<td>External F- ACTCTCTGAAGTGAGGCGCC R- CTCTTTCTCCTCAGGTCGCC</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57 °C</td>
<td>3</td>
<td>Internal F- ACATGTGACCTCCAGGCAAGAA R- CCCCCTCTCCACCTTTACAC</td>
<td>85</td>
</tr>
<tr>
<td>Ovine GRN</td>
<td>XM_004012985.1</td>
<td>57 °C</td>
<td>3</td>
<td>External F- TCAGATTACCAAGCCTTGGG A- CTCTGAGGTCAGGTACACAC</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td></td>
<td>57 °C</td>
<td>3</td>
<td>Internal F- CAGGCTTTTGCTGTAAG A- AGTCTTGTCAGGACATCT</td>
<td>130</td>
</tr>
</tbody>
</table>

*Annealing temperature

5.2.5 Quantitative RT-PCR

The homemade mix contained optimal concentrations of the essential reagents, (Invitrogen) including 10X buffer, suitable concentrations of MgCl₂, 0.2mM dNTPs, 10X SYBR green, primers (0.16μM per primer), 0.5U Platinum Taq DNA Polymerase (Invitrogen), 1μl of cDNA and water in a 15μl reaction. cDNAs of all the samples were diluted 1:5 in sterilised water and 1μl was used as the template. The quantitative RT-PCR was performed on an iCycler iQ real-time PCR detection system (Bio-Rad).

5.2.6 Western blotting

5.2.6.1 Protein extraction

Samples, 300mg, of grey matter were dissected from the frontal, parietal and occipital cortices of each animal and homogenised in 1.2ml of lysis buffer (1:5 w/v) (20mM Tris-HCl, pH 7.5; 0.15 NaCl; 0.5mM EDTA, pH 8; 5mM MgCl₂; 1% SDS; 1% Triton X-100), which contained protease inhibitors (Roche Diagnostics, IN, USA) and phosphatase inhibitors (0.1M NaF, 1mM β- glycerol phosphate, 1mM Na₃VO₄). Homogenisation was completed by passing samples through a D130 homogeniser (LabServ, Ireland) at a low speed, 15s, 4°C. Supernatants were collected and kept on ice after centrifugation (13,000rpm, 20mins, 4°C).
The pellet was resuspended in 200μl lysis buffer for a second spin and the resultant supernatant was added to the supernatant from the first spin.

**5.2.6.2 Protein electrophoresis and transfer**

As described in 2.5.2.

**5.2.6.3 Western blotting analysis**

Blots were cut horizontally midway between the 110, 60, and 42 kDa molecular weight markers and each piece was immunostained for proteins of interest within the molecular weight range (Table 5.2).

Methods of protein detection followed the practice in 2.5.3. β-actin was used as the loading control.

**Table 5.2 Antibodies used to detect PI3K I, PI3K III, Akt-Ser473 and β-actin**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Molecular weight (kDa)</th>
<th>Type of the antibody</th>
<th>Host</th>
<th>Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>p110α (PI3K I)</td>
<td>110</td>
<td>Monoclonal</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell signalling, USA</td>
</tr>
<tr>
<td>hVsp34 (PI3K III)</td>
<td>110</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>Akt-Ser473</td>
<td>60</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>β-Actin</td>
<td>42</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:30,000</td>
<td>Sigma, USA</td>
</tr>
</tbody>
</table>

**5.3 Results**

**5.3.1 Metallothionein gene expression**

The expression of the genes for the three metallothionein isoforms studied, designated as \(MT I\), \(MT II\) and \(MT III\) in the following analysis, were determined. Variable mRNA transcripts were found for ovine \(MT I\), and \(MT II\). mRNA transcripts \(MT 1A/1a\) and \(MT 2A/IIa\) were selected to represent \(MT I\) and \(MT II\) expressions. The mRNA sequence of \(MT III\) was used as a template for the quantitation of transcripts. MTs expression did not vary between the brain regions.

MT isoforms were expressed in different amounts in the control animals. \(MT I\) and \(MT III\) expressions were similar while \(MT II\) expression was less. Changes of expression of these MT isoforms in the affected brains followed a similar trend: expression slowly increased from 6 months and all had the apparent highest expression at 18 months, followed by a sharp
reduction at 24 months. The change of \textit{MT I} expression was the highest whereas \textit{MT III} expression was the least changed amongst three (Table 5.3; 5.4; 5.5)

\textbf{5.3.1.1 \textit{MT I}}

\textit{MT I} showed a high basal expression of at least 100,000 copies/µl cDNA in all the control animals. A significant increase of expression was found as early as 6 months and was highest at 18 months in the affected animals. The expression in affected brains had decreased by 24 months but remained considerably higher than that in age-matched controls (Figure 5.1A). Significant fold changes were found in the affected animals in comparison to their age-matched controls. Changes of 7, 20, 30 and 13 fold in expression between genotypes were seen at 6, 9, 18 and 24 months respectively (Table 5.3).

\textbf{5.3.1.2 \textit{MT II}}

\textit{MT II} was the least abundant isoform expressed. \textit{MT II} was expressed in all the control animals at all ages and fluctuated between ages (Fig 5.1B). A range of expression between 6x10^4 and 1.1x10^5 copy/µl cDNA was found in the controls. \textit{MT II} expression in the affected animals did not change until 6 months, after which it increased by 8 fold at 9 months, 11 fold at 18 months and 5 fold at 24 months (Table 5.4).

\textbf{5.3.1.3 \textit{MT III}}

\textit{MT III} expression was similar in the controls at all ages (Fig 5.1 C). \textit{MT III} expression was elevated relative to the age-matched controls in the affected animals from 6 months to 24 months. The up-regulation was the most significant at 9 and 18 months (P<0.01), 3 and 4 fold respectively, and 3 fold at 6 and 24 months (P<0.05) (Table 5.5).
Figure 5.1  Quantitative PCR analysis of metallothionein I (MT I) (A), metallothionein II (MT II) (B) and metallothionein III (MT III) (C) mRNA expression. *, significant difference (P < 0.05; paired t-test); **, highly significant difference (P < 0.01; paired t-test) compared to the value for control animals of the same age. Values depict the mean ± SEM for frontal, parietal and occipital brain regions for animals at each time point.
Table 5.3  Fold change of *MT I* expression

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Statistical significance*</th>
<th>Fold change in comparison to age-matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MT I</em></td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>**</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>**</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>**</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>**</td>
<td>13</td>
</tr>
</tbody>
</table>

* -, not significantly different; *, <0.05; **, <0.01

Table 5.4  Fold change of *MT II* expression

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Statistical significance</th>
<th>Fold change in comparison to age-matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MT II</em></td>
<td>2, 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>**</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>**</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>**</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.5  Fold change of *MT III* expression

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Statistical significance</th>
<th>Fold change in comparison to age-matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MT III</em></td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>**</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>**</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>*</td>
<td>3</td>
</tr>
</tbody>
</table>
5.3.2 GRN

Progranulin is encoded by GRN. The expression of GRN was at a basal mean level of 10,000 copy/μl cDNA in the controls. There was no difference in expression between the controls and the affected animals at the ages of 2 and 6 months (Fig 5.2). GRN in the affected animals was significantly up-regulated at 9 (P<0.05) and 18 (P<0.01) months, dropping a little at 24 months (P<0.01) (Fig 5.2). The fold changes in the affected animals compared to age-matched controls were 3 fold at 9 months, 5 fold at 18 months and 3.5 fold at 24 months (Table 5.6).

![GRN Graph](image)

**Figure 5.2** Quantitative PCR analysis of progranulin mRNA expression. GRN expression was similar in the affected and animals at 2 and 6 months of age, after which it increased significantly at 9 and 18 months and decreased at 24 months, remained significantly higher than the paired controls.
Table 5.6  Fold change of GRN expression

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Age (months)</th>
<th>Statistical significance</th>
<th>Fold change in comparison to age-matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRN</td>
<td>2, 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>**</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>**</td>
<td>3.5</td>
</tr>
</tbody>
</table>

5.3.3 Preliminary Western blotting on signalling transducers in the PI3K pathway

5.3.3.1 PI3K I

PI3K I expression did not show any disease-related changes (Fig 5.3).

![Western blotting analysis of PI3K I expression in the brain homogenates in the control and CLN6 affected animals of selected ages and from different regions: F, frontal, P, parietal, and O, occipital. β-Actin was the loading control.](image)

Figure 5.3  Western blotting analysis of PI3K I expression in the brain homogenates in the control and CLN6 affected animals of selected ages and from different regions: F, frontal, P, parietal, and O, occipital. β-Actin was the loading control.
5.3.3.2 **PI3K III**
PI3K III expression was detected in all the regions of the control and affected animals. However, there was no disease-related change (Fig 5.4).

5.3.3.3 **pAkt-Ser473**
Activation of protein kinase B (Akt) was recognised by dual phosphorylation at Thr308 and Ser473, designated as pAkt-Thr308 or pAkt-Ser473. pAkt-Thr308 and the total non-phosphorylated Akt were not investigated in these immunoblots, therefore the fraction of activated Akt cannot be inferred. pAkt-Ser473 showed no difference in expression between the control and the affected frontal and parietal regions (Fig 5.4).

The antibody detected a faint band below the band at the expected molecular weight (60kDa, Fig 5.4) which has been reported to appear in some blots in the customer reviews published by the company (Cat#9271, Cell signalling, USA, 2015).
Figure 5.4 Western blotting for PI3K III and pAkt-Ser473 (p473) in brain homogenates from control and the CLN6 affected animals and from regions at different ages: F, frontal, P, parietal, and O, occipital. Actin was used as the loading control.

5.4 Discussion

5.4.1 Expression of metallothioneins

Chronic and significant up-regulation of MT I and III was evident at 6 months and increased over disease development. MT II elevation was evident later, at 9 months. The up-regulation of MT I and II correlates with the induction of the proteins seen in the CLN6 affected animals at 12-14 months (Kanninen et al, 2013). Although MTs expression appeared to respond
earlier in the disease than the cytokine transcriptional factors (NF-κB, JAK/STAT, MAPK), the trend of their expression are similar to the signalling molecules investigated in the previous chapter. Changes in expression were not regional and were obvious at 6 months, highest at 18 months then declined at 24 months. These results indicate a general gene up-regulation in the affected animals. It is not understood what could have caused this similar and unanticipated generalised response in the affected animals. This generalised response follows glial activation at 2 months and correlates with the initiation of neurodegeneration at 6 months of age, but is prior to clinical disease manifestation evident at 10-14 months. It may suggest an over-drive of cellular response following glial activation and may not be causative of the neuroinflammation.

5.4.1.1 MT I and II expression in glial cells

MT I and II expression shift regional and cellular distributions during brain development. During ovine brain development, the expression is initially localised to the cells of the proliferating ventricular zones, subsequently becoming cell-specific and predominantly localised to radial glial cells, oligodendrocytes and astrocytes in the cerebral cortex. Finally the expression is exclusively found in astrocytes in the cerebral cortex, pia mater, and the choroid plexus in adult ovine brains (Holloway et al, 1997; Dincer et al, 1999). Similar MT I/II expression is reported in human brains (Blaauwgeers et al, 1993).

The up-regulation of MTs I and II has been associated with reactive astrogliosis around the injured sites in the brain, and amyloid plaque in AD (Hidalgo et al, 2001; Carrasco et al, 2006; Chung et al, 2004; 2008). The chronic and gradual up-regulation of MT I and II may correlate with glial activation in the CLN6 affected brain. However, the MT I and II expressions contrasted with the progressive and regional pattern of glial activation in that it did not differ between regions.

This lack of regional expression of MT I and II suggests a generalised response, probably in astrocytes, to the change of cellular environment. A detailed characterisation of MT I and II expression in GFAP-immunoreactive cells described by Holloway et al (1997) indicated MT I and II expression are independent of glial activation. In this study, they showed a range of immunostaining intensities for MTs, from very intense to undetectable. This suggests expression of MTs does not necessarily associate with glial activation and is likely to be responsive to other localised stimuli.
The increased expression of MT I and II may be provoked by a change of biochemical environment in CLN6 affected brains. The cytokine expression profiles revealed an abnormal inflammatory response in all brain regions. This links to a generalised disturbance of the cellular environment in the CLN6 affected brains and the MT I and II expression may reflect such a change.

5.4.1.2 MT I and II expression and metal toxicity

Another hypothesis suggests that MT I and II up-regulation is caused by the disruption of metal homeostasis in CLN6 affected brains. Concentrations of zinc and manganese were shown to increase in neurodegenerative sites in the CLN6 affected ovine brain (Kanninen et al., 2013). Similar observations, in addition to increased concentrations of copper, iron and cobalt, were found in CLN1, CLN3 and CLN5 mice models (Grubman et al., 2014). The increased expression of MTs was thought to be associated with the increase of zinc concentrations and to serve a neuroprotective role (Kanninen et al., 2013). Indeed, MTs are involved in transport and redistribution of metals in the cells (Apostolova & Cherian, 2000; Ye et al., 2001; Hao et al., 2007). Cells could take up metal bound MTs from extracellular space via endocytosis and release metal to the lysosomes, either by binding to an acceptor, being sequestered by the lower pH, and/or by the strong oxidising environment of late endosomes/lysosomes (Jiang et al., 2000; Maret, 2004; Austin et al., 2005). The unloaded MTs are either subjected to degradation in the lysosome or recycle back to the plasma membrane (Mehra & Bremner, 1985; Hao et al., 2007). Lysosomes are involved in homeostasis of biometals, and the sequestration of metals into lysosomes may be a mechanism to avoid cytosolic toxicity (Palmer, 1987; Hao et al., 2007; Lloyd-Evans et al., 2010). Induction of MTs could be caused partially by the increased concentrations of zinc and copper, as part of a protective mechanism to prevent cytosolic accumulation.

The general up-regulation of MTs without preferential distribution between the affected brain regions may indicate an increased metabolic rate, to store, donate and distribute essential metals such as zinc. This generalised response is not likely to be caused by an increased metal toxicity. The investigation of the roles of biometals in ovine CLN6 revealed a remarkable tolerance towards the high level of metal deposition in lysosomes in the CLN6 affected sheep (Palmer, 1987). Metals are more likely to be stored in the brain cells since they are post-mitotic (Palmer, 1987). There is clear evidence that the accumulation of metals
is endogenous and may not be as toxic as anticipated. Chronic copper poisoning only causes a slight elevation of brain Cu concentrations in sheep and Bedlington terriers at the haemolytic phase, suggesting a tight regulation of metal homeostasis at the BBB (Morgan, 1973; Howell et al, 1974; Herrtage et al, 1987). Another study also showed the intentional increase of ovine brain Cu concentrations did not cause neuronal damage (Dincer, 1994). Moreover, measurements of the metal contents in the CSF in the CLN6 affected brain did not reveal changes (Palmer, 1987). Thus, the higher concentration of metals may possibly arise from a high metabolic rate of the CLN6 affected brain cells and not from the disruption of metal homeostasis.

5.4.1.3 MT III expression and zinc buffering

MT III is a brain-specific protein specialising in sequestration and storage of zinc in neurons (Masters et al, 1994; Ebadi et al, 1995). A few reports demonstrated elevated expression of MT III mRNA and protein in subsets of astrocytes upon neuronal insult (Carrasco et al, 2000; Hozumi et al, 2008; Lee et al, 2010). It is difficult to interpret the expression profiles of MT III. If MT III were expressed strictly in neurons, expression may have been underestimated due to the neuronal degeneration, leading to proportionally fewer neurons in the overall cell population, hence diluted concentrations of MT III in the degenerated brains. The expression of MT III did not show a significant change at 2 months, when neuroinflammation is activated, indicating that the up-regulation of MT III is not a primary response correlating with glial activation.

Zinc is known to be involved in neurotransmission and neuromodulation, via interaction with neuronal ion channels, receptors and transporters (Cuajungco & Lees, 1997; Sensi et al, 2009). Changes of cellular composition due to neurodegeneration could alter the concentration of zinc. The expression pattern of MT III in the affected brains did not differ between regions and was similar to other MTs, showing MT III up-regulation is more likely to be attributed to a widespread up-regulation in response to glial activation.

Moreover, studies have shown that MT III expression may only play a minor role in the regulation of biologically active zinc. Reduction of MT III was found in AD patients and was thought to contribute to the pathophysiology of AD (Uchida et al, 1991; Tsuji et al, 1992). Mice with either an over-expression of MT III or a deficiency of MT III showed normal physiology and a lack of AD-like pathophysiology in spite of changes of zinc concentrations,
although the MT III deficient mice were more susceptible to the chemically induced seizures (Erickson et al, 1995; 1997). It is noteworthy that the histochemically reactive zinc, also known as the “free” zinc, which is primarily sequestered from synaptic vesicles was not altered in the MT III deficient mice (Erickson et al, 1997). Therefore, over-expression or reduced expression of MT III seems unlikely to play a primary role in pathogenesis of neurodegeneration of AD (Erickson et al, 1995; 1997). This may be the same in CLN6.

5.4.2 GRN and NCLs

The expression of GRN was not altered presymptomatically, but correlated with the development of clinical symptoms at 9 months. This suggests that a change of GRN expression is not a primary cause of CLN6 and is likely to be a response to glial activation.

The GRN homozygous mutation as a new form of NCL (Smith et al, 2012) is a premature proposal without thorough neuropathological investigation. Although similarities in clinical symptoms have been observed between GRN homozygous mutation patients and NCL patients, including progressive visual failure, convulsions, myoclonic seizures, mild cerebellar ataxia, early cognitive deterioration and retinal dystrophy (Kousi et al, 2011; Smith et al, 2012), neuropathological evidence is scarce. A neuropathological similarity, which is the fingerprint profiles in the membrane-bound structures, has been found in eccrine-secretory cells and in the endothelium from the GRN homozygous mutation patients (Smith et al, 2012).

The GRN+/- mice model has been proposed to be a model of human disease. This model has been described to have features that resemble some typical neuropathologies of NCLs, such as accumulation of intracellular autofluorescent storage material in neurons, the rectilinear profiles of those storage bodies, and increased microgliosis in the brain (Ahmed et al, 2010; Smith et al, 2012). However, overlaps of clinical symptoms and pathological features are not unusual between neurological disorders. Parkinsonism and PD have overlapping clinical symptoms, but the underlying causes can be complex (Ahlskog, 2000). Post mortem confirmed that AD patients could bear Lewy bodies and demonstrate Parkinsonian signs (Connor et al, 1998; Olichney et al, 1998). Therefore, characterisation of the neuropathological features, such as analyses of the composition in the storage inclusions in GRN homozygous mutation patients, is prudent before declaring that homozygous GRN
mutations underly a new form of NCL. Moreover, accumulation of autofluorescent lipofuscin is not an accurate phenomenon for describing the characteristic of storage bodies, which are mainly composed of protein aggregates (Palmer et al., 1986a, 1986b, 1989; Jolly et al., 1988; Palmer et al., 1992; 2013; 2015; Tyynela et al., 1993; Palmer et al., 1997; Palmer et al., 2002; Chen et al., 2004; Palmer, 2015). This characteristic is particularly important as an indication of the biochemical nature of NCL disease. One study seemingly confirmed the neurobiochemistry of GRN−/− mice is NCL-like, based on the apparent similar pathological proteins profile between Ctsd deficient mice and GRN deficient mice, (Götzl et al., 2014), but the characterisation of the protein profile does not relate to lysosomal derived organelles (e.g. subunit c).

The same study also found similar proteins stored in NCL and FTLD patients (Götzl et al., 2014). The correlation between the two diseases was then made. In the study, the human brain extract of NCL patients appeared to have increased amounts of proteins, such as TDP-43, which are associated with FTLD-TDP/GRN. In FTLD-TDP/GRN patients, there was a slight increase of saposin D (sphingolipid-activator protein D, SAP D). A few concerns are raised. Firstly, the genetic mutations were dubious in the NCL patients, with only CLN2 identified and the other NCLs were recorded as juvenile and adult NCL. Juvenile and adult NCL could be caused by many NCL genes since NCL has the characteristic of genetic heterogeneity. For instance, CLN6 NCL is reported to cause late infantile and adult forms of NCL (Kousi et al., 2011). Secondly, the study had used the brain extract from juvenile NCL patients (CLN3) and CLN2 patients. The main lysosomal storage materials are subunit c of mitochondrial ATP synthase in CLN2 and CLN3 (Kousi et al., 2011). Therefore, saposin D is not an accurate marker for proteinopathy for CLN2 and CLN3. Thirdly, and importantly, the protein extracts used for analyses in the study were not derived from the storage bodies. Lastly, TDP-43, shown to accumulate in NCLs and FTLD-TDP/GRN patients, has also been reported to accumulate in ALS patients without SOD1 mutations as well as in aged human brains (Neumann et al., 2006; Mackenzie et al., 2007; Yu et al., 2015).

The up-regulation of multiple genes at the terminal disease in the present study indicates a general metabolic overdrive in CLN6 disease. Certainly this is expected as the balance of cell type changes radically because of neurodegeneration. Therefore, potentially, a range of protein expressions could be increased in the late disease and such increases in protein...
expression are not suitable for comparison between different diseases. In this case, the proteomic methods may not be very useful. Identifying the protein in storage materials would depend on the biochemical properties of the protein and proteomics may not be ideal. Identifying the storage of subunit c has proven difficult due to its unique characteristics and special techniques are required for chromatographic isolation and mass spectral detection (Chen et al, 2004; Carroll et al, 2009; Walpole et al, 2015; Palmer, 2015). Subunit c tends to form aggregates that are insoluble to routine protein solvents and Coomassie blue staining. It requires a chymotryptic digestion rather than trypptic digestion to provide fragments for subsequent LC-MS analysis. Moreover, isolation of storage bodies could be subject to protein contamination that could lead to false positive results (Xu et al, 2010). Neurodegeneration could share similar pathological features, but the validation and interpretation of such similarities need to be more cautious and critical and should be based on a good understanding of disease pathology.

5.4.3 PI3K I is not altered in ovine NCL

There is a lack of consistent dysregulation of PI3K-Akt (Ser473) in the CLN6 affected brains, that suggests the PI3K I pathway is not likely to be correlated with the disease. The PI3K-Akt-mTOR signalling pathway has been found to be essential for normal development of the brain. Neurons are post-mitotic and over-expression of this signalling pathway would lead to detrimental effects. Mutations of signalling molecules in the pathway could lead to growth defect, including Bannayan-Riley-Ruvalcaba syndrome, megalencephaly, and hemimegalencephaly, which all share similar neuropathological phenotypes such as cortical dyslamination, cytomegaly, dysmorphic neurons and increased heterotopic neurons in the white matter (Jansen et al, 2015). Increased activity in the PI3K-Akt-mTOR pathway has also been associated with focal cortical dysplasia (FCD), which exhibits the same neuropathological characteristics as the disorders listed previously (Blümcke et al, 2011; Hauptman & Mathern, 2012). Although these neurological disorders are the extreme manifestations of intrinsic dysregulation of the PI3K-Akt-mTOR pathway in the brain, they could provide clues to the consequential events that may occur to brains if the PI3K-Akt-mTOR pathway is dysregulated. Theoretically, the early involvement of PI3K-Akt signalling pathway may affect neuronal growth during brain development. The CLN6 affected sheep grow normally until 4 months, after which the affected brain atrophies significantly.
Therefore, PI3K I-Akt may not play a major part in the pathogenesis, at least not during early development of the disease.

5.4.4 PI3K I and glucose in sheep

The PI3K I-Akt signalling pathway primarily regulates metabolism that is downstream of the insulin receptor and insulin receptor substrate (IRS) adaptor molecules in mammals, thus the pathway is particularly sensitive to glucose homeostasis (Engelman et al, 2006; Taniguchi et al, 2006). The expression of PI3K I was not disease-related, but showed variable expression between animals. This variable expression may relate to the different metabolic rates or states of individual animals. There is a dietary influence on PI3K-Akt signalling, which may affect the expression of PI3K. Fasting of animals before sacrifice was suggested to preclude such an influence (personal communication, Dr Greg Smith and Dr Hayden McEwen, Auckland University).

During starvation, insulin is not necessary for glucose disposal because bodies are subject to hypoglycaemia, thus fasting should be useful to eliminate effects of glucose on insulin-PI3K signalling. However, the regulation of metabolism in ruminants is fundamentally different from that in non-ruminants. Unlike non-ruminants, the energy supply to ruminants arises primarily from short-chain volatile fatty acids (VFA) produced by the fermentation of microbial activity in the rumen (Lindsay, 1959; Brockman, 1978). Ruminants rely on gluconeogenesis from noncarbohydrate sources for glucose and have the greatest rate of gluconeogenesis during starvation. Short-chain VFAs, such as propionate, butyrate and valerate are more potent stimuli for insulin production (Brockman, 1978). Fasting of ruminants may not be useful as the microbial activity could remain active to produce VFA and potentially lead to production of insulin thereby affecting PI3K signalling.

Acquisition of glucose in the CNS is mainly dependent on the non-insulin sensitive glucose transporters such as GLUT-1 (astrocytes), GLUT-3 (neurons), and GLUT-5 (microglia) (McEwen & Reagan, 2004). Insulin appears to be less necessary for glucose utilisation for the CNS. However, it has been reported that insulin-PI3K signalling may play a role in neuronal survival, synaptic and dendritic plasticity, learning and memory, and neuronal circuitry formation in the CNS (Banks et al, 2012). Insulin and IGF-I receptors, to which insulin binds, are widely expressed in brain, especially in regions associated with olfaction, appetite, and
autonomic functions (Havranksa, et al., 1978; Werther et al., 1987; Unger, et al., 1989; Wozniak et al., 1993), suggesting roles for insulin in these specific regions.

Early studies on the distribution of insulin receptors (IRs) in rat brain demonstrated only moderate expression of IRs in the cerebral cortex (Werther et al., 1987), and the immunohistochemical investigation of the downstream signalling molecules Akt-Ser437 showed only mild immunostaining in the cerebral cortex of ovine brain in the CLN6 affected and the control animals (preliminary observation), indicating a less significant role of insulin signalling in the cerebral cortex, perhaps less tightly regulated than in the brain region which have higher expression of IRs. Therefore, the insulin-PI3K signalling in the cerebral cortex may not be directly involved in the disease mechanism.

5.4.5 PI3K I and TFEB

Although dysregulation of PI3K-Akt signalling was not found in the current study, manipulation of PI3K-Akt-mTOR signalling may be of interest for treatment. Activation of the transcription factor EB (TFEB) has been found to increase lysosomal biogenesis which improves the lysosomal clearance in pathological conditions in lysosomal storage diseases (LSDs) (Sardiello et al., 2009; Medina et al., 2011). TFEB is regulated by mTORC1 signalling, which lies downstream of PI3K signalling. mTORC1 localises to the cytoplasmic surface of late endosome/lysosomes where it phosphorylates TFEB in Ser211, the phosphorylated TFEB then interacts with 14-3-3 protein, masking its nucleus translocation signal (NLS) and thus retaining the TFEB(Ser211)/14-3-3 complex in the cytoplasm (Rocznia-Ferguson et al., 2012; Martina et al., 2012). Up-regulation of 14-3-3 was found in the CLN5 affected sheep brain, specifically in the cerebral cortex, hippocampus, thalamus, brainstem and cerebellum (Janet Xu, personal communication, Lincoln University), indicating an increased inhibition of lysosomal biogenesis. A possible therapeutic manipulation may target the PI3K-Akt-mTOR pathway to increase the lysosomal biogenesis.

Future experiments would be required to confirm the observations from the current study of PI3K signalling pathway, and examine the expression of 14-3-3 and phosphorylated TFEB in the CLN6 affected brains. However, the initial results from the current study and available published studies suggest little significance of the PI3K pathway in the CLN6 disease, thus it is not worthwhile studying this pathway further.
5.4.6 PI3K III is not altered

PI3K III expression is found in the cerebral cortex but is not correlated with the disease. In the previous chapter, BDNF and Trk B have been shown not to be affected in the CLN6 sheep. The vesicle formation, sorting and trafficking did not appear to be disrupted in ovine CLN6. Moreover, the storage materials in lysosome derived organelles in NCLs, except CLN1 and 10, consists mainly subunit c of mitochondrial ATP synthase (subunit c) and normal lysosomal components, suggesting a specific defective processing of subunit c in the endocytic pathway.

5.5 Conclusion

Gene expressions of metallothioneins and progranulin follow a similar pattern to other genes investigated in the previous chapter, indicating a wide-spread gene up-regulation paralleled or followed by glial activation. The up-regulation of MTs may act as an adaptive mechanism against an increased metabolism in CLN6 affected cells. A colocalisation study of MTs and glial markers could be further investigated to understand if the up-regulation of MTs are correlated with glial activation. If glial markers do not colocalise with MTs and MTs show immunoreactivity in all glial cells, it would mean a discrete pathway triggers glial activation regionally that sets it apart from the regulation of some genes, such as progranulins, which are up-regulated non-selectively for the increased metabolic demand of glial activation.

Preliminary investigation of the PI3K pathway revealed a lack of correlation with the disease development. The current observation for the PI3K signalling pathway could be validated but further studies of this pathway may not be productive.
6.1 Summary and general discussion of findings

This thesis aimed to study the role key inflammatory mediators could play in ovine CLN6 disease pathology, as well as the possibility of identifying therapeutic targets. To summarise, the neuronal ceroid lipofuscinoses (NCLs, Batten disease) are a group of fatal inherited human neurodegenerative diseases affecting an estimated 1:12,500 live births worldwide. They are defined by common clinical features, including blindness and seizures; and uniform neuropathological features, which include lysosomal accumulation of fluorescent storage material, with defining histochemical properties and ultrastructure, as well as profound neurodegeneration and widespread neuroinflammation within the CNS (Palmer et al, 2013). This group of disease is caused by mutations in at least 13 different genes, which include 8 classical NCL genes and 4 newly proposed NCL genes (http://www.ucl.ac.uk/ncl/mutation.shtml). The disease also occurs in animals and this study is based on ovine CLN6, ideal for study of the human CLN6 disease.

The underlying pathogenesis of the disease remains poorly understood. In the 1980s, peroxidation was proposed to be the major cause of the neuropathology. This idea originated from lipofuscin-like storage bodies found in lysosomal derived organelles in affected individuals. The storage materials were initially thought to be a cross-linking product between protein and peroxidised lipids (Chio et al, 1969a, b). Hence, lipid peroxidation was proposed to be the cause of the formation of storage bodies. However, the compositional analyses of the isolated storage bodies in 1980s showed a predominant and abnormal storage of the subunit c of the mitochondrial ATP synthase and a normal lipid composition of lysosome (Palmer et al, 1986a; b; 1988; 1989a; 2002; 2015). The fluorescence of storage bodies probably arises from the protein aggregates (Palmer et al, 2002). The biochemical analyses of the stored subunit c revealed a normal gene expression in the affected animals, and the accumulated subunit c is a mature protein which has been processed into the inner mitochondrial membrane prior to lysosomal accumulation (Palmer et al, 1990; 1992; Medd et al, 1993; Chen et al, 2004; Walpole et al, 2015; Palmer, 2015). This indicates a defective pathway involving the turnover of mitochondrial subunit c.
Other hypothesis followed to suggest the genetic mutation leads to disruption in lysosomal function, autophagy and mitochondrial turnover and degradation, and in turn to the accumulation of the storage bodies (Seehafer & Pearce, 2006). These were thought to cause further cellular disturbances, which subsequently aggravates the cumulative process in lysosomes and increases the oxidative stress in lysosomes. The oxidative stress was thought to be facilitated by the presence of transitional metals in lysosomes and to cause mitochondrial dysfunction (Brunk & Terman, 2002; Seehafer & Pearce, 2006). This scenario led to a number of studies in vitro to prove oxidative stress plays a role, in the context of ER stress (Kim et al, 2006b; Wei et al, 2008), autophagy disturbances (Vidal-Donet et al, 2013), mitochondrial abnormalities (Majander et al, 1995; Dawson et al, 1996; Siakotos et al, 1998) and lysosomal dysfunction (Prasad et al, 1996).

The facts strongly argued against this hypothesis. The stored lipid profiles are normal in the storage bodies (Palmer et al, 1986a; b; 1988; 1989a; 2002; 2015). The storage bodies were enriched with transitional metals and the enrichment correlated with the tissue specificity of the metal metabolism (Palmer, 1987). The presence of metals did not facilitate lipid peroxidation since there was no evidence of lipid peroxidation (Palmer, 1987; Palmer et al, 1988; Jolly et al, 2002). Moreover, the stored protein is highly specific and unique (Palmer, 2015). In subunit c storing NCLs, storage is specific to subunit c and subunit c storage is specific to the NCLs, and none of the other 16 ATP synthase subunits or any other inner mitochondrial membrane proteins are stored (Fearnley et al, 1990; Chen et al, 2004; Palmer, 2015). Some subunit c accumulation has also been reported in other LSDs, like Niemann-Pick types A and C, GM1 and 2 gangliosidoses and MPS I, II and III, but the degree of storage is minimal in these diseases and less uniform than in NCLs, suggesting that the accumulation of subunit c is especially amplified in lysosomes of neuronal and non-neuronal cells in subunit c storing NCLs (Elleder et al, 1997). Moreover, the accumulation of the storage bodies occurs in nearly all cells of NCL patients, and cells in the CNS do not degenerate equally. Some neurons with a heavy burden of storage bodies survive, suggesting the accumulation is not damaging but is a separate consequence of the genetic lesion (Palmer et al, 2002; Oswald et al, 2005).

The post-translational modification of subunit c may offer some clues as to what may cause the abnormal turnover of subunit c. The trimethylation of lysine43 in the subunit c is
conserved among Metazoans and is suggested to associate with cardiolipin to facilitate formation of the subunit c ring structure in the inner membrane of mitochondria (Watt et al., 2010; Walpole et al., 2015). The stored subunit c in lysosomal derived organelles is also trimethylated (Chen et al., 2004; Walpole et al., 2015). The abnormal subunit c storage is unlikely to arise from dysfunctional mitochondria. Despite the conflicting reports on mitochondrial dysfunction (Seehafer & Pearce, 2006), evidence showed normal mitochondrial activities in the CLN6 affected sheep (Palmer et al., 1992). There was also a lack of disease-induced mitochondrial activities as the expression of mitochondrial proteins (MnSOD and COX IV) were not increased in the CLN6 brain (Chapter 3). Although there is a lack of clear correlation between the trimethylation of the subunit c and its storage in lysosomes, it may be possible that the subunit c trimethylation or demethylation, mediated by methyltransferases (Walpole et al., 2015), may be altered in the sorting and trafficking pathway for subunit c and lead to pathological accumulation, in which all NCL proteins play a role.

Neuroinflammation plays a central role in the neuropathology of NCLs (Palmer et al., 2013). That neuroinflammation preceded neurodegeneration was established in studies of the CLN6 sheep model. Both astrocytic and microglial activation began perinatally, spreading from specific foci in cortical regions associated with later neurodegeneration and symptoms (Oswald et al., 2005; Kay et al., 2006; 2011). Glial activation is often associated with the release of ROS and production of pro- and anti-inflammatory cytokines (Brown & Neher, 2010; Glass et al., 2010). Thus, glial activation may trigger oxidative stress. The up-regulation of MnSOD, reported to be significant in CLN6 affected human fibroblasts and in the brains of CLN6 affected sheep, was thought to be associated with glial activation related oxidative stress and/or neuroinflammatory cytokine-regulated responses (Fridovich, 1983; Merrill & Benveniste, 1996; Sayre et al., 1999; Heine et al., 2003). This prompted the present study to observe any change of MnSOD expression in CLN6 over time, as well as a study of the expression of other oxidative stress related mediators, including HO-1 and iNOS (Schipper et al., 2009a;b; Brown & Neher, 2010). iNOS has been proposed to be expressed in activated microglial cells and to be responsible for release of pathological NO (Chapter 3; Dringen, 2005; Saha & Pahan, 2006; Pautz et al., 2010; Brown & Neher, 2010). As reported in Chapter 3, the expression of MnSOD was not correlated with the disease development, iNOS was not
expressed in sheep and HO-1 expression was low. These results clearly indicate that oxidative stress does not contribute to the progression of the disease, nor in the mechanism by which cytokines induce neurodegeneration, and revealed a problem associated with sampling brain regions.

Neurodegeneration and glial activation lead to major cytoarchitectural changes in the brain. False positive results could be produced when isolating specific isolation of brain regions for observation. Expression of mitochondrial proteins, MnSOD and COX IV, are typical examples (Chapter 3). Immunohistochemistry showed that the expression of those markers and the distribution of mitochondria is highly uneven (Fig 3.5; Fig 6.1). This is even more pronounced in the affected brains (Fig 3.5; Fig 6.1). The atrophy of the cortex in the affected animals resulted in a greater density of MnSOD and COX IV expressing cells in some locations, leading to a misconception of an increased number of such cells in these locations in the affected animals when observation was confined to a microscopic area (Fig 6.1). This could explain the conclusion that the expression of these proteins increased in a small region as well as the higher gene expression for MnSOD in parieto-occipital cortices at late disease (Heine et al, 2003; Chapter 3). However, integration of the MnSOD and COX IV expressing cells in the overall neurodegenerative cortices showed that the overall protein expression is reduced as these cells are subject to neurodegeneration. This means that sampling a small region randomly for a study may not represent the change of the whole brain since neurodegenerative events do not occur equally in all cells nor in all locations.
Figure 6.1  A representative figure of sampling that could be acquired for different experiments.

This figure illustrates the problem associated with sampling, which is discussed in section 4.4.2. The area in the blue box indicates the area that would be sampled to acquire the amount of tissue for used in for the RNA and protein extractions. A heterogeneous cell population stained for MnSOD is distributed unevenly across the cortical layers of the control parietal cortex (left). The sampling area would need to be altered to compensate for the loss of cells in the affected parietal cortex (right), in order to match the overall cell number in control samples. Because the immunoreactive cells in the atrophied cortex are relatively more packed in comparison to the controls, more MnSOD mRNA
could be obtained than from the control for the same amount of cells harvested. The area in the red box indicates the same area for microscopic observation. The similar location in the control is replicated in the affected, disregarding the cytoarchitectural change caused by the neurodegeneration in the area. This can lead to an appearance of increased MnSOD expression in cells, when, in fact, it is a phenomenon of compression of the cell layers that has changed the distribution of cells.

The iNOS-centred mechanism of inflammation and oxidative stress should be discarded from the neuroinflammatory cascade in ovine and human NCL. Expression of iNOS has been largely recognised and discussed as a major driving cause of oxidative stress following microglial activation in rodent models (Mander & Brown, 2005) and also in many other published studies. However, the evidence is clear that iNOS is not expressed in sheep (Chapter 3), or in humans (Gross et al, 2014). This illustrates that using rodent models for inflammatory studies may not be ideal and the results derived from rodent models may not be translatable to humans.

Meanwhile, a long term trial of therapeutic intervention by minocycline, a broad spectrum anti-inflammatory drug, which has activity against neuroinflammation (Kumar et al, 2003; Nikodemova et al, 2007), did not result in inhibition of neuroinflammation or neurodegeneration (Kay & Palmer, 2013). Early administration prior to the onset of neurodegeneration and effective drug absorption into the brain did not aid the suppression (Kay & Palmer, 2013). This shows that non-selective drug intervention to suppress neuroinflammation is not likely to succeed. High throughput drug screening methods are not practical in large animal models. A mechanism-based choice of drugs for inhibition may hold some hope in the suppression of neuroinflammation. Thus, defining the relevant neuroinflammatory cascade was thought more likely to provide a more accurate target and time-span in which therapy will be required for optimal benefit.

The loss of neuronal populations in ovine CLN6 was characterised as progressive, selective, regional and cell- type specific, all well as predicted by glial activation (Oswald et al, 2005; 2008; Kay et al, 2011). Thus it was anticipated that molecular profiles would vary between brain regions, and changes to these molecular cues would possibly parallel neuroinflammation prior to neurodegeneration in the parieto-occipital cortex (Oswald et al,
A longitudinal molecular dissection of the neuroinflammatory cascade was initiated through a study of pro- and anti-inflammatory cytokines (TNF-α and IL-1β, and TGF-β and IL-10) and their co-stimulated pathways, including a positive feedback loop which can result in neuronal damage (JAK/STAT, NF-κB and MAPK) and a negative feedback loop which leads to inhibition of pro-inflammation (SOCS3) (Chapter 4; Glass et al., 2010; Barry, 2011). Other positive feedback pathways that aggravate neuronal damage were investigated, including the loss of neurotrophic factor support due to dysfunction in the GERL pathway (BDNF and TrkB) and the activation of adaptive immunity that results in a breach of the BBB and entry of lymphocytic cells (CD4 and CD8) (Henderson et al., 2000; Kopan et al. 2004; Lim et al., 2007; Saha et al., 2012). Other makers proposed to be involved in the pathogenesis of NCLs, namely, PI3K I, PI3K III, metallothioneins and progranulin were also investigated in Chapter 5.

An unanticipated inflammatory response was revealed. Changes in the gene expression were widespread in the diseased brain, contrasting with the progressive and regional involvement of glial activation, and there was a lack of evidence for unique disease-causative lesions. The gene expression of the neuroinflammatory mediators described in Chapters 4 and 5 display a remarkably similar trend of elevation in expression following glial activation and a sharp decrease in the dying brain. Additionally, the proposed transcriptional factors responsible for these cytokine expressions were either activated in the advanced disease (NF-κB, MAPK) or activated moderately (STAT1 and 3) (Raivich et al., 1999; Li & Verma, 2002; Kumar et al., 2003; Brown & Neher, 2010; Minogue et al., 2012). Only one anti-inflammatory modulator is actively up-regulated prior to neuroinflammation, SOCS3 (Baker et al., 2009). A disruption of the blood-brain barrier was discounted as there was no sign of lymphocytic infiltration in the brain of ovine CLN6 (Chapter 4), while roles for the pro- and anti-inflammatory cytokine regulated pathways remain (Figure 6.2). In general a large number of gene expressions change in some way with the disease, but most, if not all, are unlikely to be central to the disease pathogenesis.

The changes in gene expressions are widespread in the affected CLN6 brains. In conclusion, the current molecular approach of dissecting of neuroinflammatory cascade did not provide sufficient information for selection of therapeutic targets but showed that a complicated neuroinflammatory network underlies the CLN6 disease. This reinforces the need for
understanding the neuroinflammation in a genome-wide scale using a genetic screening method with carefully selected regions.

The approach of assembling “pathways” from simple models, which focus on a few pathways, timepoints and/or single cell types, is simplistic. There are many weaknesses associated with simple disease models. High dosages of inflammatory stimuli are often applied to cell cultures to provoke intensive responses and the measurement are usually focused on a few inflammatory markers. Studies of neuroflammation on acute disease models, such as brain trauma or on the human tissue extracted from the end-point disease, represent severe biochemical responses but lack resolution since the neurodegeneration is chronic in nature and gradually evolves with time.

The heterogeneity of cell populations and the distribution of the cells in different brain regions may lead to different survival rates. Loss of GABAergic interneurons in the ovine CLN6 brain displayed a contrasting pattern in different regions, emphasising that cellular location and interconnectivity are the major determinants of neuron survival (Oswald et al, 2008). Subtle molecular cues may be localised, and not be detected by the current method, yet may contribute to the vulnerability of cells. Therefore, even though they did not show regional differences, subpopulations of cells and other determining factors may make them more vulnerable to inflammatory-induced decline (Barry, 2011). Hopefully, the genetic screening method is able to provide some insights into these determining factors.

The CNS is a hierarchically organised complex system, divided into brain regions, cell types, organelles and molecular pathways. A systemic network approach would allow integration of multi-level information, such as transcriptomic and proteomics, to identify causal molecular drivers of cellular, circuit-level and brain-wide pathology in disease (Parikshak et al, 2015). Methods such as RNA-sequencing is able to quantify levels of transcripts and their isoforms in a biological sample, which could be specific cells or tissue, at a specific developmental stage or physiological condition (Section 6.1.1.1; Wang et al, 2009). A preliminary study using RNA-sequencing conducted on the CLN6 affected Merino and control sheep showed it is possible use in the study of disease mechanisms in the NCLs (Chang et al, 2014). Preliminary gene ontology analysis associated differential gene expression patterns with the induction of inflammation, cell surface receptor linked signal...
transduction, cellular and metal ion transport, tissue remodelling (activation and inactivation of genes associated with actin, myotubules, cell-adhesion) and apoptosis (Chang et al., 2014). The results highlight the diverse molecular changes accompanying neurodegeneration. The costs associated for using such technology have become much lower than previously and the technologies are now readily accessible. Future studies could use high read depth RNA-Seq analyses in ovine NCL models to develop a better understanding of the pathogenic cascade in different NCL variants and for multiple tissue comparisons, and to evaluate the effects of gene therapy agents.

On a separate notion, the weaknesses of determining a disease phenotype without an thorough understanding of the biochemical lesions was discussed in Chapter 5. Although the genotype and phenotype correlations in NCLs are not clear, the classical forms of NCLs (CLN1, 2, 3, 5, 6, 7, 8 and 10) have uniform pathological features, particularly the storage of specific proteins, either subunit c of mitochondrial ATP synthase or sphingolipid-activator proteins A and D (SAP A and D). These abnormal storage materials are not a result of a general metabolic disruption in the GERL pathway. For example, PI3K I and PI3K III were not altered and BDNF and Trk B did not play roles in disease development (Chapters 4 and 5), suggesting the general endocytic pathways is not disrupted in early CLN6 disease. Thus, the specificity and uniqueness of the protein storage suggest mutations of classical NCL genes, which may be categorised as the subunit c storing type or the SAP A and D storing type, arising from pertubations of a common and specific biochemical pathway in which NCL proteins may interact. For instance, CLN5 has been suggested to interact with CLN3 in SH-SY5Y human neuroblastoma cells in a quantitative proteomics study (Scifo et al., 2013). Although the idea that the NCL proteins lie on the common pathway is an assumption, it at least presents a collective identity that unites NCLs. More recently proposed NCL forms lack such biocmetal characterisation. The expression of the progranulin gene (GRN) was not altered presymptomatically in the ovine CLN6 brain (Chapter 5). This reduces the possibility of progranulin having a common association with NCLs. The study which characterised the pathobiochemical features shared between NCLs and FTLD (Götzl et al., 2014), was largely based on inappropriate methods due to a lack of understanding of biochemical pathology of
NCLs. There is not enough information on some of the more recently classified NCLs to be sure that they really are NCLs.

**Figure 6.2** Updated neuroinflammatory pathway in ovine CLN6 Batten disease

### 6.1.1 Future directions

#### 6.2.5.1 Transcriptomics and validation

As mentioned previously, pursuing expression of the individual gene becomes futile against the background of the complexity of brain biochemistry and a wider screening method for genes responsible for the disease mechanism is required, potentially by genome-wide screening. Among the many potential techniques, transcriptomics is sensitive, informative, reproducible, and cost-effective. Transcriptomics could analyse a large number of genes for primary screening and the determination of molecular relationships and networks (Wang et al, 2009; Kavanagh et al, 2013). These could be validated by qPCR and protein analyses. Transcriptomics studies would allow measurement of the precise levels of transcripts and their isoforms within specific brain regions at different stages of disease. The technique could be applied preferentially to the brain at an early stage of the disease, during which the cytoarchitecture is little altered.

Pathway analysis in conjunction with RNA-Seq could help to select genes from RNA-Seq using a fold change cut-off, generally a two-fold difference with p<0.05, categorising the significantly changed genes into functional pathways using a reference database (Emmert-
Streib & Glazko, 2011; Khatri et al, 2012). These pathways can then be ranked according to the number of genes in the pathway that are differentially expressed (Kavanagh et al, 2013). The results may enable further functional inferences and relevant studies to be developed. However, analysis of the significance of the pathway needs to be considered since some genes may play roles in networks affecting multiple pathways (Kavanagh et al, 2013).

Single cell transcriptomics are also available (Kanter & Kalisky, 2015). The technique would help to identify and characterise molecular profiles in specific cell types and allow a better understanding of a particular cell response under the disease influence. The method may detect changes of gene expression that are unique and disease-associated but masked by the “bulk” tissue analysis (Kanter & Kalisky, 2015). Laser-capture microdissection could be adapted to isolate specific cells of interest from microscopic regions of tissue that could later be subject to RNA-Seq for characterisation. This method is particularly useful for complex and heterogeneous tissue (Emmert-Buck et al, 1996; Hodges et al, 2006).

6.2.5.2 Proteomics of signalling molecules

The signalling transduction system relies greatly on the half-lives of the proteins and of the post-translational modification, especially phosphorylation. Thus, the maintenance of signalling transduction may rely on the half-lives of the signalling molecule and detecting the activation of a signalling pathway requires detection of the post-translationally modified transducers.

Protein and mRNA synthesis, mRNA transcription and translation, protein and mRNA degradation may all be regulated co-ordinately to deliver either stable or transient production, and amplification of the protein (section 4.5.7). The correlation between protein expression and mRNA expression seems to be reflected in the half-life of a protein. For instance, short-lived protein expression, such as for signalling molecules, correlates tightly with mRNA expression (Wang et al, 2002b; Yang et al, 2003; Raj et al, 2006; Sharova et al, 2009; Dey et al, 2015). On the other hand, the transient activation of a signalling pathway could occur in the absence of an increase of mRNA transcripts. The signalling molecules investigated in the present study were mainly determined at the transcriptional level and may represent a chronic activation of transcriptional pathways but not temporal or transient activation. For instance, the pre-existing cytosolic fraction of transcriptional factors could be
activated rapidly, either by phosphorylation (e.g. JAK) or dissociation from an inhibitor (e.g. NF-κB). This allows an instant response and reduction of transcriptional noise (Raj & van Oudenaarden, 2008). Therefore, signal transduction could possibly be sustained independently of mRNA expression in a short-term if a protein has a relatively long half-life. In this case, mRNA expression may not predict the activation of the signal transduction.

It has been found that the pattern of gene transcription is discontinuous, a phenomenon sometimes referred as a transcriptional burst, and the corresponding protein expression seems to correlate to such pattern (Chubb et al, 2006; Raj & van Oudenaarden, 2008). One of the proposed mechanisms for the transcriptional burst is the existence of pre-initiation complexes that bind to the promotor region of DNA and enable multiple rounds of RNA polymerase II transcription (Blake et al, 2003; 2006). This would mean that gene expression by transcriptional factors does not necessarily require an increased mRNA transcription of the transcriptional factors, but that is relies more on the stability of an mRNA molecule.

Detection of the phosphorylation state of transcription factors, their upstream regulators or the downstream substrates by Western blotting is more appropriate than detection of mRNA expression. For instance, activation of JAK/STAT3 could be detected by the expression of phospho-STAT3 (Tyr 705). This would avoid the uncertainty of the mRNA-protein expression correlation and could be used to validate the results generated from transcriptomics. However, the function of signalling molecules can be pleiotropic. For example, the cause and function of the sole elevation of SOCS3 prior to neuroinflammation and neurodegeneration are not understood when its known activator, IL-10, and transcription factor (STAT3) were not elevated until later in the disease progression (Chapter 4; Yasukawa et al, 2003; O'Shea & Murray, 2008; Qin et al, 2008; Baker et al, 2009). The anti-inflammatory role of SOCS3 is also ambiguous. It has been proposed to have an anti-inflammatory role, but it clearly did not supress neuroinflammation in this study despite its statistically significant up-regulation. IL-6 is known as an inflammatory cytokine. SOCS3 plays a role in the self-inhibiting mechanism of IL-6 signalling, in which SOCS3 expression is induced by IL-6-STAT3 activation to dampen the inflammatory action of IL-6, via SOCS3 binding to the tyrosine 757 of the IL-6 receptor/gp130. Sustaining STAT3 activation in addition to deletion of the SOCS3 binding site on IL-6 receptor (gp130) demonstrated an anti-inflammatory action of IL-6 identical to that of IL-10 (Yasukawa et al 2003; El Kasmi et al,
In this case, SOCS3 seems to be pro-inflammatory, suggesting that SOCS3 could play pleiotropic roles in neuroinflammation. Thus, evidence of the activation of one gene could be insufficient to predict the biochemical consequence of that gene. Quantitative proteomic analysis of phosphorylated proteins may improve the understanding of the signalling network.

6.2.5.3 Comparison between South Hampshire and Merino ovine CLN6 and Borderdale CLN5

Different CLN6 mutations occurred naturally in Merino and South Hampshire sheep. The mutation in CLN6 in South Hampshire comprises a 402bp deletion and a 1bp insertion that includes the 83bp sequence of the complete first exon; whereas a different mutation (c.184C>T in exon 2) in CLN6 in the affected Merino sheep causes a major amino acid exchange (p.Arg62Cys) (Section 1.5). Ovine CLN5 disease in Borderdale sheep has also been well characterised (Jolly et al., 2002; Frugier et al., 2008). The disease-causing mutation is a substitution at a consensus splice site (c.571+1G>A), leading to the excision of exon 3 and a truncated putative CLN5 protein (Frugier et al., 2008). Comparative studies of the neuropathology in CLN6 in Merino and South Hampshire sheep and CLN5 in Borderdales are underway and the comparisons between these different ovine models will follow.

The clinical signs and neuropathology reported are remarkably similar in all these affected sheep in spite of the differences in the mutation types and genes (Jolly et al., 1982; 1989, Mayhew et al., 1985, Cook et al., 2002; Jolly et al., 2002; Oswald et al.; 2005; Tammen et al., 2006; Kay et al., 2006; Frugier et al., 2008; Palmer et al., 2015). These affected sheep exhibit clinical symptoms including behavioural changes, blindness and motor deficits. Subunit c of ATP synthase specifically accumulates in most cells in most tissues in both CLN5 and CLN6 NCL, and many other forms (Section 1.4.1; Palmer, 2015). Brain atrophy accompanies neurodegeneration and neuroinflammation in these affected sheep. The disease with ovine models of CLN5 and CLN6 resemble the human diseases. In fact, remarkably uniform pathological features are also shared between forms of NCLs and the predicted location of NCL proteins in the GERL pathway (Palmer et al., 1986b; 1989; 1992; Fearnley et al., 1990; Cooper et al., 1999; Oswald et al., 2005; 2008; Pontikis et al., 2004; Bible et al., 2004; Kay et al., 2006; 2011; von Schantz et al., 2009; Schmiedt et al., 2012) suggesting a common pathogenic
pathway contributes to all NCLs. Thus, the study of ovine models could provide some insights into pathogenesis in all NCL forms in animals and humans.

6.3 Conclusion

The observed neuropathology of human NCL represents the end result of a cascade of events. A dissection of the molecular cascade in this longitudinal study aimed to provide a more accurate target and time-span in which therapy will be more likely to be effective. The neuroinflammatory gene expression profiles in the ovine CLN6 affected brains presented here revealed a widespread up-regulation of genes following neuroinflammation and discounted oxidative stress as part of the disease mechanism. Cytokine-mediated neuroinflammation remains and SOCS3 was the only molecule found to be significantly up-regulated prior to neurodegeneration and concurrent to the glial activation. However, the regulatory role of SOCS3 is not understood. These results indicate that a complex signalling network underlies a primary defect in the NCLs. In addition, correct sampling of a targeted region for study is important as neurodegeneration and glial activation can change the cytoarchitecture and this change could differ in cell types and brain regions. Studies of underlying molecular cascades by transcriptomics with carefully selected target regions in ovine models would be beneficial for inferring the function of CLN6 and CLNS as well as understanding their impact on the biochemistry in the brain. Hopefully, the study could be translated into human disease, and will benefit the understanding of the pathogenic cascade in different NCL variants and also the correlation between different NCL genes that may likely converge in the same biochemical pathway. Moreover, a better understanding of the pathogenic cascades can provide possible targets for therapeutic intervention to ameliorate disease.
Postscript

This postscript describes some additional experiments performed after the completion of this thesis. MnSOD expression was determined by immunohistochemistry of sagittal CLN6 affected brain sections and matched controls over the span of disease development, 2, 6, 9, 18 and 24 months. Adjacent sections were immunostained for COX IV. The results are to strengthen the argument discussed in chapter 3 with oxidative stress is not likely to play a major role in disease mechanism, based on a lack of activation of oxidative stress markers throughout the disease progression. The immunostaining for MnSOD and the mitochondrial marker, COX IV, showed paralleled staining in all the brain regions and revealed a regionally specific reduction in cerebral cortex. Severe neurodegeneration leads to a dramatic change in the cytoarchitecture of cortical grey matters, causing MnSOD and COX IV immunoreactive cells, which are distributed across the cortical layers II-VI, to compress to form two layers at the boundaries between layers I and II and between layers IV and V in the affected cortices at both 18 and 24 months. This phenomenon was not observed in non-degenerative brain regions. It was a consistent observation in the animals and a statistical analyses support the observation. A larger sample size was included for immunohistochemical experiment (n=3) and the immunostaining of MnSOD and COX IV in different cortical regions was quantified and analysed. Statistical analyses confirmed the observation found in this thesis and the results presented in this postscript and the thesis have been combined for publication.
Figure 1  Quantitation of immunoreactivity of MnSOD and COX IV in different brain regions. Thresholding image analysis of immunostaining in the cortical grey matter layer II-VI in different brain regions. Results are the means of at least 3 cortical columns from each region and expressed as the % of pixels of digitalized microscopic images that stained above a threshold intensity, vertical bars represent the standard error of the mean. *, significant difference (P < 0.05; paired t-test); **, highly significant difference (P < 0.01; paired t-test).
Table 1  Proportional decrease of MnSOD and COX IV immunostaining in the CLN6 affected cortical layers II-VI at 18 and 24 months. Quantitative immunostaining are presented as % of immunostaining in comparison to the matched control regions (n=3).

<table>
<thead>
<tr>
<th>Proteins</th>
<th>MnSOD</th>
<th></th>
<th></th>
<th>COX IV</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18m</td>
<td>SEM</td>
<td>24m</td>
<td>SEM</td>
<td>18m</td>
<td>SEM</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>75</td>
<td>11</td>
<td>69</td>
<td>14</td>
<td>76</td>
<td>41</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>54</td>
<td>11</td>
<td>39</td>
<td>7</td>
<td>56</td>
<td>15</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>60</td>
<td>12</td>
<td>33</td>
<td>5</td>
<td>63</td>
<td>35</td>
</tr>
</tbody>
</table>
Quantitation of immunoreactivity of MnSOD and COX IV in cortices. This is the combined data from the three regions (frontal, parietal, occipital). Thresholding image analysis of immunostaining in cortical layers II-VI in brain regions. Results are the means of sampled cortical columns and expressed as the % of pixels of digitalized microscopic images that stained above a threshold intensity, vertical bars represent the standard error of the mean. *, significant difference (P < 0.05; paired t-test); **, highly significant difference (P < 0.01; paired t-test).
Table 2  Proportional decrease of MnSOD and COX IV immunostaining in the CLN6 affected cortical layers II-VI at 18 and 24 months. This is the combined data from three regions. Quantitative immunostaining are presented as % of immunostaining in comparison to the controls (n=3).

<table>
<thead>
<tr>
<th>Proteins</th>
<th>MnSOD</th>
<th>COX IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/Brain Regions</td>
<td>18m</td>
<td>SEM</td>
</tr>
<tr>
<td>Affected cortex</td>
<td><strong>63</strong></td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3  Ratio of the proportional decrease of immunostaining area between MnSOD and COX IV in the CLN6 affected cortical layers II-VI at 18 and 24 months (n=3). The ratios indicate a correlated reduction of mitochondria and MnSOD expression.

<table>
<thead>
<tr>
<th></th>
<th>MnSOD/COX IV at 18 months</th>
<th>MnSOD/COX IV at 24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>0.99</td>
<td>0.93</td>
</tr>
<tr>
<td>Parietal Cortex</td>
<td>0.96</td>
<td>1.05</td>
</tr>
<tr>
<td>Occipital Cortex</td>
<td>0.95</td>
<td>1.1</td>
</tr>
<tr>
<td>Whole Cortex</td>
<td>0.97</td>
<td>1</td>
</tr>
<tr>
<td>SEM</td>
<td>0.008</td>
<td>0.036</td>
</tr>
</tbody>
</table>
Appendix A

1 Gel electrophoresis and Western blotting reagents

15% Resolving Gel: 1.9ml 40% acrylamide: bisacrylamide (29:1 w/w)
1.25ml 1.5M Tris pH 8.8
50μl 10% LDS
1.8ml H₂O¹
25μl 10% ammonium persulphate (APS, Pharmacia Biotech, Uppsala, Sweden)
2.5μl Tetramethylethlenediamine (TEMED, BDH)

4% Stacking Gel: 0.25ml 40% acrylamide: bisacrylamide (29:1 w/w)
0.63ml 0.5M Tris pH6.8
25μl 10% LDS
1.6ml H₂O¹
12.5μl 10% APS
2.5μl TEMED

Electrophoresis Buffer: 25mM Tris
192mM glycine
0.1% LDS

Transfer Buffer: 25mM Tris
192mM glycine
20% methanol

2 10 X TBE
54g Tris base
27.5g Boric acid
20ml 0.5M EDTA pH 8.0
In 1L total H₂O¹

3 SOC medium
2g Bacto-tryptone
0.5g Yeast extract
1ml 1M NaCl
0.25ml 1M KCl
In 97mls H2O¹
Autoclaved and cooled to RT
1ml sterilised 2M Mg²⁺ stock
1ml sterilised 2M glucose
Up to 100ml with H2O¹, pH 7.0

4 LB agar
10g NaCl
10g Tryptone
5g Yeast extract
20g Agar
Up to 1L with H2O¹, pH 7.0
Autoclave

5 LB broth + ampicillin
5g NaCl
10g Tryptone
5g Yeast extract
Up to 1L with H2O¹, pH 7.0
Autoclave
100µg/ml Ampicillin

1 Water deionised by electrodeionization was used for all experiments described in this thesis.
References


consensus classification proposed by an ad hoc Task Force of the ILAE Diagnostic Methods Commission. Epilepsia, 52, 158–74.


Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar
degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration.

Campbell IL. (2005). Cytokine-mediated inflammation, tumorigenesis, and disease-
associated JAK/STAT/SOCS signalling circuits in the CNS. Brain Res Brain Res Rev, 48,
166-77.

Candotti F, Oakes SA, Johnston JA, Gilliani S, Schumacher RF, Mella P, Fiorini M, Ugazio AG,
Badolato R, Notarangelo LD, Bozzi F, Macchi P, Strina D, Vezzoni P, Blaese RM, O'Shea

Cannella B, Raine CS. (2004). Multiple sclerosis, cytokine receptors on oligodendrocytes

Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid

Distinct early molecular responses to mutations causing vLINCL and JNCL presage ATP
synthase subunit C accumulation in cerebellar cells. PLoS One, 6, e17118.

Neuroscience, 143, 911-22.

Metallothioneins are upregulated in symptomatic mice with astrocyte-targeted

factor-alpha type 1 receptor deficient mice reveal a role of IL-6 and TNF-alpha on brain


Cherry JD, Olschowka JA, O'Banion MK. (2014). Neuroinflammation and M2 microglia, the good, the bad, and the inflamed. *J Neuroinflammation*, 11, 98.


Cooper JD. (2003). Progress towards understanding the neurobiology of Batten disease or neuronal ceroid lipofuscinosis. *Curr Opin Neurol, 16*, 121-8.


Haan C, Heinrich PC, Behrmann I. (2002). Structural requirements of the interleukin-6 signal transducer gp130 for its interaction with Janus kinase 1, the receptor is crucial for kinase activation. *Biochem J, 361*, 105–11.


Kay GW, Palmer DN. (2013). Chronic oral administration of minocycline to sheep with ovine CLN6 neuronal ceroid lipofuscinosis maintains pharmacological concentrations in the brain but does not suppress neuroinflammation or disease progression. *J Neuroinflammation, 10*, 97.


Lee J, Duan W, Mattson MP. (2002). Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. *J Neurochem*, 82, 1367-75.


Minogue AM, Barrett JP, Lynch MA. (2012). LPS-induced release of IL-6 from glia modulates production of IL-1β in a JAK2-dependent manner. *J Neuroinflammation, 9*, 126.


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Palmer DN, Oswald MJ, Westlake VJ, Kay GW. (2002). The origin of fluorescence in the neuronal ceroid lipofuscinoses (Batten disease) and neuron cultures from affected sheep for studies of neurodegeneration. *Arch Gerontol Geriat*, 34, 343-57.


VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J Cell Biol, 113*, 1203–12.


peritoneal macrophage inflammatory profile in cirrhosis depends on the alcoholic or hepatitis C viral etiology and is related to ERK phosphorylation. *BMC Immunol, 13, 42.*


Woodall CJ, Maclaren LJ, Watt NJ. (1997). Differential levels of mRNAs for cytokines, the interleukin-2 receptor and class II DR/DQ genes in ovine interstitial pneumonia induced by maedi visna virus infection. *Vet Pathol, 34*, 204-11.


