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Location and connectivity determine GABAergic interneuron survival in the brains of South Hampshire sheep with CLN6 neuronal ceroid lipofuscinosis

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Abstract

The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are fatal inherited neurodegenerative diseases. Sheep affected with the CLN6 form provide a valuable model to investigate underlying disease mechanisms from preclinical stages. Excitatory neuron loss in these sheep is markedly regional, localized early reactive changes accurately predicting neuron loss and subsequent symptom development. This investigation of GABAergic interneuron loss revealed similar regional effects that correlate with symptoms.

Loss of parvalbumin positive neurons from the affected cortex was apparent at four months and became profound by 19 months, as was somatostatin positive neuron loss to a lesser extent. Conversely calbindin and neuropeptide Y positive neurons were relatively preserved and calretinin staining temporarily increased. Staining of subcortical regions was more intense but subcortical architecture remained relatively intact. Discrete subcortical changes followed from cortical changes in interconnected regions. These data highlight cellular location and interconnectivity as the major determinants of neuron survival, rather than phenotype.

Keywords

Batten disease; neuronal ceroid lipofuscinosis; CLN6; GABAergic interneurons; ovine model; lysosomal storage disease

Introduction

The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are inherited neurodegenerative diseases that manifest in progressive psychomotor retardation, blindness and premature death

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(Goebel et al., 1999; Haltia, 2003; 2006). Collectively they are the most common cause of progressive childhood encephalopathy (Cooper, 2003), mutations in at least eight genes, *CLN1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, *CLN7/MFS8*, *CLN8* and *CLN10/CTSD*, giving rise to overlapping clinical forms (www.ucl.ac.uk/ncl) formerly classified by the age of onset of symptoms.

Three of these genes, *CLN1*, *CLN2* and *CTSD*, encode soluble lysosomal hydrolases (Vesa et al., 1995; Sleat et al., 1997; Siintola et al., 2006) and *CLN5* a lysosomal glycoprotein of unknown function (Holmberg et al., 2004; Sleat et al., 2005). The other genes encode predicted transmembrane proteins of unknown function. *CLN3* may code for a lysosomal membrane protein (Ezaki et al., 2003; Kyttälä et al., 2004) or a resident of pre-lysosomal vesicular transport compartments (Fossale et al., 2004). The *CLN8* protein may recycle between the endoplasmic reticulum (ER) and ERGolgi intermediate compartments (Lonka et al., 2004). *CLN6* encodes an ER resident protein that modulates the endocytosis of exogenous proteins (Gao et al., 2002; Wheeler et al., 2002; Heine et al., 2004; Mole et al., 2004), while *CLN7* also encodes a putative membrane protein (Siintola et al., 2007).

Severe brain atrophy and the accumulation of fluorescent lysosome-derived storage bodies in neurons and most other cells throughout the body are the pathological hallmarks of the NCLs (Goebel et al., 1999; Haltia, 2003; 2006). Defects in *CLN2*, *CLN3*, *CLN5*, *CLN6* and *CLN8* are all associated with the abnormal specific accumulation of subunit c of mitochondrial ATP synthase in lysosome derived storage bodies (Chen et al., 2004; Herva et al., 2000; Palmer et al., 1992, 1997; Tyynelä et al., 1997). The pathogenic mechanisms underlying these devastating neurodegenerative diseases are poorly understood and current treatments limited to alleviating symptoms. Descriptions of human pathology are largely confined to autopsy samples that reveal little about the development of disease while recent magnetic resonance imaging studies (Pěna et al., 2001; Vanhanen et al., 2004) lack the resolution to describe cellular and molecular events.

The progression of pathogenesis is more easily studied in animal models and a range of genetically engineered and spontaneously occurring mouse models are available (Mitchison et al., 2004). Large naturally occurring animal models are particularly suited because their CNS is more like that of humans, and the development of pathology and symptoms more similar to the human diseases (Tammen et al., 2006; Frugier et al., 2008). The South Hampshire sheep model is particularly well characterised (Jolly et al., 1989; Palmer et al., 1992; Oswald et al., 2005; Kay et al., 2006; Tammen et al., 2006). Linkage studies placed the genetic lesion in these sheep on chromosome 7q13-15, syntenic with the human *CLN6* location on chromosome 15q21-23 (Broom et al., 1998; Gao et al., 2002; Wheeler et al., 2002). Quantitative PCR studies showed that *CLN6* expression is severely reduced in them, but the underlying mutation has yet to be determined. It is probably a novel non-coding mutation in a regulatory region, which may have human analogs (Tammen et al., 2006).

To gain insights into the pathogenic mechanisms we have been studying progressive pathological changes in the CNS of *CLN6* affected sheep and recently showed that marked reactive changes occur long before clinical symptoms become apparent (Oswald et al., 2005). Abnormal activation of astrocytes was already evident prenatally and activated microglia shortly after birth (Kay et al., 2006). These early glial changes were remarkably localized, occurring first in regions associated with the much later atrophy and the development of clinical symptoms (Oswald et al., 2005), a feature also noted in mouse models of various NCLs (Mitchison et al., 2004; Cooper et al., 2006; Kielar et al., 2007; Partanen et al., 2008).

Populations of GABAergic interneurons are consistently affected in human NCLs (Williams et al., 1977; Tyynelä et al., 2004) and in mouse models (Bible et al., 2004; Cooper et al.,

1999; Mitchison et al., 1999; Pontikis et al., 2004; Kielar et al., 2007). Studies of CLN6 affected sheep suggested selective effects upon populations of parvalbumin containing cortical interneurons (Oswald et al., 2001). A regional and time dependent decline in γ -amino butyric acid (GABA) concentrations was notable among a number of changes revealed in a metabolomic study of affected sheep brains (Pears et al., 2007). Here we report a comprehensive survey of interneuron phenotypes in CLN6 affected sheep at different stages of disease. This revealed selective effects upon interneuron populations that differ markedly between locations within the brain. These findings emphasise that cellular location and connectivity are much more important determinants of neuron survival than phenotypic identity.

Materials and Methods

Animals

Affected New Zealand South Hampshire lambs were bred, maintained and diagnosed as described (Tammen et al., 2006). All animal procedures accorded with NIH guidelines and the New Zealand Animal Welfare Act, 1999.

Tissue preparation and immunohistochemistry

Immunohistology was performed on brains from animals aged 12 days, and 2, 4, 6, 12, and 19 months, together with unaffected Coopworth controls as before (Oswald et al., 2005). Brains were perfusion-fixed at post-mortem with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4, left in fixative for 48 h at 4°C, then equilibrated in cryoprotectant (15% sucrose, 30% ethylene glycol, in 0.05M PBS, pH 6.8) and stored at -130°C. Subsequently 50 μ m sagittal sections were cut through one hemisphere with a freezing microtome and collected into 96 well plates containing cryoprotectant.

Matched series of sections selected at previously defined levels 2 and 4 (Oswald et al., 2005), approximately 13.3 mm and 6.7 mm from the midline of adult control brains, were immunostained for calcium binding proteins or neuropeptides normally expressed by subclasses of GABAergic interneurons (Freund and Buszaki, 1996; DeFelipe, 1997). Following quenching of endogenous peroxidase activity with 1% hydrogen peroxide in 50% methanol, sections were blocked in 15% normal goat serum and incubated overnight, 4°C, in monoclonal mouse anti-parvalbumin (1:5,000, Swant, Bellinzona, Switzerland), rabbit anti-calretinin (1:5,000, Swant), rabbit anti-calbindin-D28K (1:2,000, Swant), rabbit anti-somatostatin (1:2,000, Peninsula Laboratories, San Carlos, CA), or rabbit anti-neuropeptide Y (1:2,000, Chemicon, Temecula, CA). Horseradish peroxidase based detection of antibodies used Vectastain Elite ABC kit reagents (Vector Laboratories, Burlingame, CA) or Sigma (St Louis, MO) biotin and extravidin conjugated reagents. All antibodies and reagents were diluted in 1% goat serum in PBS, pH 7.4, containing 0.04% Thiomersol (Sigma), and 0.2% Triton X-100. Sections were mounted in a solution of 0.5% gelatine and 0.05% chromium potassium sulphate on glass slides (Milton Adams Ltd., Auckland, New Zealand), air-dried, dehydrated through increasing alcohol concentrations, cleared in xylene, and coverslips mounted with DPX (BDH Chemicals, Poole, UK).

Microscopy and image analysis

Photomicrographs were taken in an inverted DMRB microscope (Leica, Wetzlar, Germany) with differential interference contrast enhancement, and a Spot RT colour digital camera (Diagnostic Instruments, Sterling Heights, MI). A Zeiss Axioskop 2 MOT microscope with an Axiocam digital camera was used for overlapping images for photomontages using Axiovision 4.5 software (Carl Zeiss UK Ltd., Welwyn Garden City, UK). Mounted sections were scanned

on a flatbed scanner (HP5400c, Hewlett-Packard, Palo Alto, CA) at 600 pixels per inch. Figures and photomontages were prepared in Photoshop CS3 (Adobe Systems Inc., San Jose, CA).

Areas of specific neuron cell bodies within the parieto-occipital cortex at level 2 were measured using Stereo Investigator 4.33a (MicroBrightField Inc., Williston, VT) with the computer display superimposed on the actual image viewed through a Zeiss Axioskop 2 MOT microscope, using the Lucivid system (MicroBrightField Inc.), and a x100 oil immersion objective (1.4 NA). Somata areas were calculated by the software after tracing the cross-sectional area of individual cells (Cooper et al., 1999). A total of 80-100 individual neurons were sampled for each antigen for each brain, selected at random across the whole parieto-occipital cortex. Soma areas of at least 200 Neuro Trace labelled pyramidal neurons within the parieto-occipital layer III were determined, using the same imaging setup in epifluorescence viewing mode, and a rhodamine filter set.

Immunoreactive neurons within the parieto-occipital cortex at level 2 were counted in 10 different fields, using a 10x objective with a viewing area of 3.14 mm² per field. The standard deviation of these counts across different fields was within 15 % of the mean.

Western blot analysis

Samples, 30 mg, dissected from specific regions of 6 and 20 month old control and affected sheep brains frozen immediately at post mortem were homogenised and sonicated in 0.5 ml of PBS containing 1% lithium dodecyl sulphate and 1mM dithiothreitol. The protein concentrations of supernatants after centrifugation (12,000g, 20min, 4 °C) were measured with bicinchoninic acid (Pierce Biotechnology, Inc., Rockford, IL) and proteins in samples, 5 µg protein/well, separated by electrophoresis as described (Frugier et al., 2008). Proteins were transferred to Hybond C-extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) at room temperature in a Bio-Rad Mini Trans-Blot electrophoresis cell, in 25 mM Tris, 192 mM glycine and 20% methanol, 1h, 100V.

Membranes were cut in two between the 31 and 45 kDa molecular weight markers and immunostained for tubulin and calretinin with rabbit anti-calretinin (1:2,000; Swant) and mouse anti-βIII tubulin (1:40,000; Promega, Madison, WI) using appropriate secondary antisera and enhanced chemiluminescence horseradish peroxidase detection (Amersham). The resultant films were scanned on a flatbed scanner (HP5400c, Hewlett-Packard) and Quantity One image analysis software (BioRad, Hercules, CA) was used to quantify individual bands. Background was determined from five unstained areas on each film. The calretinin to tubulin band intensity ratio was determined for each sample, and the means for each region and age group calculated.

Results

This study of regional disease related changes in the distribution of interneuron populations from 12 days to mature disease revealed a distinctive pattern of change for each calcium binding protein. Loss of neurons positive to parvalbumin from the affected cortex became apparent at four months of age and had become profound by 19 months. The extent of loss varied markedly between regions, as to a lesser extent did the loss of somatostatin positive neurons. Conversely calretinin, calbindin and neuropeptide Y positive neuron populations remained well preserved, or in the case of calretinin, increased until mature disease. These effects upon interneuron survival were most profound in cortical regions even though staining for these markers was most intense in subcortical regions (Fig. 1).

Cortical changes

Cerebral cortex—The characteristic laminar pattern of parvalbumin staining (Figs. 1A, 2A and 3A) was already established in the parietal and other regions of the cerebral cortex of both affected and control brains at 12 days of age, with positive neurons and basket-like profiles abundant in layers III-V in most cortical regions, along with some lighter stained cells with pyramidal morphologies. Progressively fewer parvalbumin-positive interneurons were present in the affected parieto-occipital and occipital cortex from four months and few remained at 19 months (Figs. 1A, 2A and 3B). This neuron loss was accompanied by a decrease in the density and number of parvalbumin-positive fibres and axon terminals, although some positive fibres were still evident in cortical layer I and the white matter of affected sheep at 19 months. Axon terminal basket-like cell body outlines could be seen, albeit faintly, in the affected cortex (e.g. arrows, Fig. 3B), as the obscuring neuropil staining became less intense.

NeuroTrace-stained layer III pyramidal neuron somata in the parieto-occipital cortex were measured as a representative excitatory neuron population, revealing a shift to smaller cell size in affected sheep that became more apparent with disease progression (Fig. 4A). The opposite trend was apparent in parvalbumin positive interneurons in the parieto-occipital cortex of affected sheep which displayed a marked age-related shift towards larger cell size (Fig. 4B). Counts of parvalbumin positive interneurons in the affected sheep parieto-occipital cortex revealed 80 neurons per field (3.14 mm^2) at 12 months, compared to 180 neurons per field in the age-matched control sheep, indicating that smaller parvalbumin positive cells were lost preferentially.

In contrast to parvalbumin staining, calretinin immunoreactivity was relatively increased in the affected brain at all ages, and was particularly pronounced in ventral regions of both affected and control brains (Fig. 1B). Darker neuropil staining and increased detection of calretinin immunoreactive Cajal-Retzius cells were apparent within neocortical layer I (Fig. 2B). The subventricular zone of severely affected sheep stained more darkly with calretinin than the subventricular zone of control sheep, notably a discrete band of immunoreactivity above the dorsal border of the lateral ventricle, seen most distinctly at level 4 (Fig. 1B). Counting calretinin positive interneurons in the parieto-occipital cortex of sheep at 12 months of age yielded 25-30 neurons per field from affected brains compared to 15 cells per field in control sheep. Also in contrast to parvalbumin positive cells, a lower proportion of calretinin positive interneurons in the parietal cortex of affected sheep at 19 months had large somata (Fig. 4C), but the larger calretinin positive interneurons were largely retained in other cortical regions.

These immunohistochemically observed changes in calretinin concentrations in different regions were verified by Western blotting of the ligand, normalised to β -III-tubulin to represent the total number of neurons (Fig. 5). There was six-fold more calretinin per neuron in the visual cortex of affected 6 month old brains than in controls, four-fold more in the affected somatosensory cortex, three-fold more in the affected motor cortex and two-fold more in the affected hippocampus. These increases over controls became less pronounced at 20 months of age, returning to near control values.

Calbindin immunoreactivity was macroscopically similar in the affected and control cortex, except for a darker rim of calbindin staining at the pial surface and more intense staining of some frontal and occipital lobe regions in affected brains at 19 months (Fig. 1C). Calbindin staining also highlighted the discrete band of calretinin positive neurons in the subventricular zone of mature affected brains (Fig. 1C). Immunoreactive interneurons appeared to be relatively well preserved in affected sheep (Fig. 2C) and numerous calbindin positive Cajal-Retzius like neurons were present in the outer half of the molecular layer, towards the pial surface in both control and affected brains. Neuron counts revealed a small increase in the number of calbindin positive neurons in the parieto-occipital cortex of affected brain at 12

months of age, 50 positive neurons per field, compared to 40 per field in control sheep. However, this apparent increase may be due to the compression of cortical layers in the affected brain. Cross sectional area measurements revealed fewer large cells in affected brain (Fig. 4D).

Somatostatin immunoreactivity was similar in the neocortex of affected and control sheep until 12 months of age, but fewer positive interneurons were evident in the parieto-occipital cortex of affected sheep by 19 months, only 15 somatostatin positive interneurons per field, compared to 80 per field in both affected and control sheep at 12 months of age. There was a less pronounced loss of these neurons from the somatosensory cortex and immunoreactive neurons were relatively preserved in the affected frontal cortex. Cross sectional areas of immunopositive cells were similar in control and affected brains at all ages (Fig. 4E).

Neuropeptide Y positive interneurons were scarce, but largely preserved, in all cortical subfields of the affected sheep at 19 months, and positive cell soma were scattered across all layers. Cross sectional somata measurements suggested even fewer neuropeptide Y positive neurons with large and complex cell body morphologies in the mature affected cortex (Fig. 4F).

Entorhinal Cortex—Degenerative changes took longer to become apparent in the entorhinal cortex. The loss of parvalbumin positive interneurons and their processes did not become apparent until a year and it took 19 months for loss from the olfactory cortex to be noticed. The principal cell layers of the affected sheep entorhinal cortex were compressed at 19 months (Fig. 6A), notably layer III where a reduction of calretinin immunoreactivity was apparent in pyramidal neurons from 6 months. Conversely diffuse calretinin staining of the neuropil in lower layers of entorhinal cortex was enhanced in affected sheep at 19 months of age.

Projection neurons in layer II of the affected entorhinal cortex stained weakly for calbindin at 12 and 19 months of age, and the number of immunoreactive interneurons and bipolar cells appeared to be reduced (Fig. 6B). No differences were observed in somatostatin immunostaining of affected and control entorhinal cortex, even at 19 months of age.

Hippocampal changes

Disease related changes in the hippocampus (Fig. 7A) were more subtle than those in the cerebral cortex, but followed the same trends during disease progression.

Parvalbumin, calbindin and neuropeptide Y containing neurons became depleted to varying degrees as the disease advanced, but calretinin and somatostatin positive neurons largely persisted.

Parvalbumin positive interneurons in the hippocampus included distinctly stained multipolar and basket-like cells in the polymorphic and granule cell layers of the dentate gyrus, stratum oriens and the pyramidal cell layer in CA1-CA3 (Figs. 7B, 8A and 9A). Loss of parvalbumin-positive cells from the affected hippocampus was evident by 6 months (Fig. 7B) and by 19 months few remained, particularly in CA1 (Fig 8A). Horizontally orientated interneurons in the stratum oriens were common amongst the persisting immunoreactive cells (Fig. 8A).

Calbindin immunoreactivity was more intense in dentate granule neurons of affected sheep at 6 and 12 months of age than in controls (Fig. 8B), as was staining of their projections into the stratum lucidum of CA3 (Fig. 9B). However by 19 months very few interneurons were stained in CA1-3 of affected sheep. The number of calbindin-positive Cajal-Retzius like cells along the hippocampal fissure in affected sheep at 12 and 19 months was increased compared to controls (Fig. 8B).

Somatostatin immunoreactivity was relatively well preserved in the hippocampus of affected sheep, even at advanced stages of disease (Figs. 8C and 9C). Immunoreactive cells in the polymorphic layer of the dentate gyrus and in the stratum oriens of CA1 of affected sheep appeared to be hypertrophic (Fig. 8C). A reduction in neuropeptide Y immunoreactive axon terminals and dendrites in the hippocampal formation of affected sheep apparent at 12 months was pronounced in the polymorphic layer of the dentate gyrus and the pyramidal cell layer and stratum lucidum of CA3 by 19 months (Figs. 8D and 9D).

Subcortical nuclei

In general staining of subcortical interneurons was much stronger than staining of cells in cortical regions (Fig. 1). Staining of subcortical regions of normal brains for parvalbumin and calbindin was intense and extensive, and many areas stained heavily for calretinin. However unlike those of the cortex the immunoreactivities of these markers were largely unaltered by disease progression and the gross architecture of the subcortical regions remained relatively intact. Changes associated with the disease were less pronounced, were localised and occurred later. There was some regional loss of parvalbumin and calretinin positive neurons, but immunoreactivities for somatostatin and neuropeptide Y remained unchanged.

Parvalbumin immunoreactivity in interneurons, projection neurons, and diffuse neuropil staining in subcortical nuclei of affected brains were comparable to those of control brains at one year, but by 19 months parvalbumin immunoreactivity had become reduced in the reticular thalamic nucleus, substantia nigra reticulata, the ventral posterior thalamic complex, subthalamic nucleus, and zona incerta of affected brains (Fig. 1A). In contrast, interneuron morphology and density remained normal in the affected striatum and the intense parvalbumin immunoreactivities in the lateral dorsal geniculate nucleus, superior colliculus and optic tract remained.

Calretinin immunoreactivity of subcortical regions appeared to complement the profile of parvalbumin staining, highlighting a series of different nuclei. In general the pattern of subcortical calretinin immunoreactivity was unaffected by disease, but there were some significant changes with disease progression, some beginning at a relatively early age. Loss of immunoreactive thalamic projection neurons from the visual relay nucleus (lateral dorsal geniculate nucleus, Fig. 1B), first evident at six months, was marked at 12 months. Calretinin positive neurons were largely absent from the superior colliculus by 19 months while afferent fibres were relatively preserved (Fig. 10A). Diffuse immunoreactivity of the neuropil and the number of calretinin positive cells in the visual association nuclei (lateral dorsal and lateral posterior thalamic nuclei) of affected sheep were reduced at this age (Figs. 1B and 10B). Calretinin positive neuron loss from the affected reticular thalamic nucleus had become apparent at this age (Fig. 1B), the zona incerta had a thinner appearance and its calretinin positive cell density appeared to be reduced. Calretinin immunoreactivity remained intense in a variety of subcortical nuclei, notably the amygdaloid nuclear group and the optical tract, and calretinin positive interneurons remained dispersed throughout the striatum (Fig. 1B). Immunostained projection cells were still apparent in the substantia nigra at 19 months, but fewer neurites and fibres stained compared to controls (Fig. 10C).

The intensity of calbindin immunoreactivity within subcortical nuclei was much higher than in any cortical or hippocampal sub-region (Fig. 1C). This intense immunoreactivity was largely retained with disease progression and at 19 months immunostaining of the lateral dorsal geniculate nucleus appeared to be enhanced, whereas that of the superior colliculus was reduced. Calbindin immunoreactivity changed little in other subcortical areas of the affected brain, including the intense staining of the striatum, where the medium-sized spiny projection neurons and a profuse network of processes in the neuropil were stained. The basal forebrain nuclear complex, the optic tract and the substantia nigra all stained darkly.

Cerebellar changes

Parvalbumin and calbindin immunostaining of cerebellar basket and Purkinje cells was relatively unchanged in affected sheep brains, even at 19 months of age. Concentrations of calretinin in the affected cerebellum remained normal with time (Fig. 5).

Discussion

This systematic analysis of GABAergic neurons in the CLN6 affected sheep confirmed marked differences in the survival of different subtypes and between different brain regions during disease progression, as suggested in a preliminary study (Oswald et al., 2001). Parvalbumin containing interneuron loss from the affected cerebral cortex began earlier and was more pronounced than changes in populations of calretinin, calbindin and somatostatin immunoreactive interneurons (Fig. 1). Even more pronounced than these cell-type-specific effects were the differences between cortical regions, in both the extent and rate of interneuron loss. Moreover, marked differences in interneuron survival between cortical and subcortical structures in affected sheep closely mirror the relative sequence of changes in early glial activation in these regions (Oswald et al., 2005). Cellular location and connectivity are the overriding influences upon the timing and severity of neuron loss during NCL pathogenesis. This extends beyond excitatory neuron populations, to include the selective loss of GABAergic interneurons characteristic of these disorders.

These interneurons form three separate populations based on their immunoreactivities to parvalbumin, calretinin, and somatostatin/calbindin (DeFelipe, 1997), but there is some overlap. Parvalbumin has been identified as a marker of fast-spiking large basket and chandelier local circuit neurons that exert a powerful influence on pyramidal activity in the cerebral cortex (DeFelipe, 1997; Markram et al., 2004) and hippocampus (Freund and Buzsáki, 1996). In the rat, somatostatin defines a population entirely separate from parvalbumin and calretinin containing neurons (Kubota et al., 1994). Calbindin immunoreactivity is also observed in pyramidal cells and a small proportion of parvalbumin and calretinin immunoreactive cells (Kubota et al., 1994; DeFelipe, 1997; Gonchar and Burkhalter, 1997). Calretinin is a marker for dendrite targeting neurons with bipolar and bitufted cell morphologies that form vertical projections across all cortical layers and somatostatin mainly marks Martinotti cells that form wide-ranging inhibitory connections with pyramidal dendrites in layer I (DeFelipe, 1997; Markram et al., 2004). Nearly 90% of somatostatin immunoreactive neurons also colocalise with calbindin and about one third of somatostatin immunoreactive cells contain neuropeptide Y (Kubota et al., 1994).

Selective effects upon subpopulations of GABAergic interneurons

The data extend findings that subpopulations of GABAergic interneurons are affected in human and murine NCLs, to different extents depending on the calcium binding proteins or neuropeptides that these neurons usually express (Tyynelä et al., 2004; Mitchison et al., 2004), and are consistent with a metabolomic investigation of affected sheep brains (Pears et al., 2007) which showed that GABA concentrations declined in a regional manner with disease progression, initially from the frontal and occipital regions in line with the loss of GABAergic neurons.

The regional pattern of parvalbumin positive neuron loss from affected sheep brains paralleled that of pyramidal neuron loss which is associated with the development of clinical symptoms (Oswald et al., 2005). Somatostatin positive interneuron loss followed the same regional trend but later in disease progression, loss from the occipital cortex being most pronounced and loss from the parietal or frontal cortex less evident. Changes in neuropeptide Y positive interneurons

closely mirrored these changes, in keeping with the colocalization of somatostatin and neuropeptide Y in a subset of rat cortical interneurons (Kubota et al., 1994).

In contrast, the number of calretinin containing interneurons increased in the parieto-occipital cortex of affected sheep, and peaked coincident with the progression from regionally specific to generalised atrophy of the cerebral cortex, as shown by immunohistology (Fig. 2) and confirmed by Western blotting (Fig. 5). A similar rise and then decline to normal staining was noted in calbindin positive dentate granule neurons (Fig. 8B), and in their CA3 stratum lucidum projections (Fig. 9B). The relative overall long-term preservation of calretinin positive interneurons is a general feature evident in different human (Tyynelä et al., 2004), and murine NCLs (Bible et al., 2004; Pontikis et al., 2004), but the rise and decline in calretinin expression during disease progression has not been documented before. It may represent an attempted neuroprotective response, to buffer fluctuations in intracellular calcium concentration, as has been suggested in primary neuron cultures (D'Orlando et al., 2002).

These marked differences in GABAergic neuron survival in different cortical and subcortical structures, despite their common embryological origin (Parnavelas, 2000; Marin et al., 2000; Xu et al., 2003) and similar morphology (Kawaguchi et al., 1995), indicate that more than suggested differences in the long-term ability of calcium binding proteins to buffer against excitotoxicity determines selective neuron vulnerability (D'Orlando et al., 2002). For example, the relative preservation of parvalbumin positive striatal neurons, compared to the pronounced loss of their cortical counterparts (Fig. 1), argues against a generalized vulnerability. Such contrasting patterns of GABAergic interneuron loss in different brain regions are not consistent with a widespread metabolic defect that preferentially affects GABAergic cells, or makes them more vulnerable to accumulating storage products as previously suggested (Walkley et al., 1995). Regional functionality and connectivity appear to be better determinants of neuronal susceptibility to degeneration, also indicated by data from a study of the excitatory neuron populations (Oswald et al., 2005).

This also appears to be the case in human NCLs where severity of interneuron loss correlates well with the total neuronal loss in different hippocampal subfields (Tyynelä et al., 2004). Parvalbumin and somatostatin containing neurons in NCL mouse models are generally more affected than those staining for calretinin (Cooper et al., 1999; Mitchison et al., 1999; Bible et al., 2004; Pontikis et al., 2004). Data from *CLN6* deficient *nclf* mutant mice reveal regional and cell type-specific effects upon interneuron survival (Cooper et al., unpublished observations). These studies also reveal marked differences in interneuron survival between different cortical layers or hippocampal subfields, consistent with the concept that information flow through these networks may be compromised selectively in each form of NCL.

Functional implications of interneuron changes

Local interneurons of the cerebral cortex are implicated in integrating, coordinating and sharpening the response properties of principal cell types (Wehr and Zador, 2003; Trevelyan and Watkinson, 2005), and the loss of specific interneuron subpopulations is likely to have significant functional consequences. In line with this a general slowing of the rhythmic electroencephalogram activity and an increase in slow oscillatory activity during wakefulness occurs in most forms of NCL, including *CLN6* (Haltia, 2003; Mole et al., 2005). These oscillations are thought to have a role in attention and perception (Singer, 1999; Engel et al., 2001), and to be synchronized by electrically-coupled fast-spiking GABAergic interneuron networks (Tamás et al., 2000; Hasenstaub et al., 2005; Sohal and Huganard, 2005). Since the leading cortical neuropathology in the affected sheep affects pyramidal neurons and parvalbumin immunoreactive fast-spiking interneurons, it is likely that the progressive loss of these neurons increasingly disrupts the γ -frequency oscillations (30-80 Hz) associated with an activated cortical network in the normal brain.

Interneuron dysfunction or loss may also predispose cortical networks towards seizure activity (Schwaller et al., 2004; McCormick and Contreras, 2001), a prominent feature of the NCLs (Haltia, 2003). The apparent loss of somatostatin immunoreactive interneurons from the affected sheep neocortex correlates well with the onset of absence seizures at 19 months of age (Mayhew et al., 1985), and may be mechanistically related. Furthermore, a selective and time-dependent loss of calretinin and somatostatin containing interneurons is associated with the development of generalized cortical seizures in mice lacking the transcription factor DLX1 (Cobos et al., 2005), and spontaneous seizures only occur in CLN1 mice once significant loss of these neurons is underway (Kielar et al., 2007). The differential survival of calcium binding protein immunoreactive neurons in Creutzfeldt-Jakob disease (Ferrer et al., 1993; Guentchev et al., 1997) is accompanied by severe alterations in cortical network activity that may directly contribute to epileptogenesis (Ferrer et al., 1993; Wieser et al., 2006). A down-regulation of parvalbumin expression rather than the actual loss of hippocampal basket and chandelier cells may occur in temporal lobe epilepsy (Sloviter et al., 2003), and similar 'phenotypic silencing' has been observed in the *mnd* mouse model of CLN8 variant late infantile NCL (Cooper et al., 1999).

Characteristic spike and wave activity in absence seizures depends on recurrent connections between GABAergic neurons in the reticular thalamic nucleus and thalamocortical relay cells (Steriade et al., 1993; Fuentealba and Steriade, 2005). Sensory relay and reticular thalamic neurons are both affected before seizure onset in CLN1 mice (Kielar et al., 2007). Likewise parvalbumin and calretinin immunoreactivities were reduced in both sensory relay and reticular thalamic nuclei in the affected sheep by 19 months, the age at which absence seizures first appear (Mayhew et al., 1985). Similar effects were also evident in the zona incerta, which provides direct inhibitory inputs to the neocortex, and indirect inputs via relay neurons in intralaminar thalamic nuclei (Barth et al., 2002). Since zona incerta neurons exert a modulating influence over wide regions of the cerebral cortex, loss of these neurons may help to tip the affected neocortex towards a hyperexcitable state.

Interconnectivity and defects in network activity

Despite heavier staining of subcortical regions, attributable in part to calcium binding protein expression by distinct populations of projection neurons as well as interneurons on this region (Celio, 1990), generalised subcortical neuropathology is evident only late in the disease and changes can be correlated to earlier pathology in neocortical regions, and to the development of clinical symptoms. Atrophy starts earlier and progresses faster in the primary visual and parieto-occipital cortices than in the somatosensory, primary motor, frontal association, and entorhinal cortices of affected sheep and subcortical brain pathology is delayed (Oswald et al., 2005). Subcortical interneuron loss was similarly delayed. For instance loss of calretinin immunoreactive thalamic projection neurons from the visual relay lateral dorsal geniculate nucleus, (Fig. 1B) was first evident when microglial activation became apparent in this nucleus, at six months. In line with the delayed onset of neuropathology in the somatosensory cortex, neuron loss from the somatosensory relay ventral posterior thalamic complex was delayed until 19 months. Also delayed to this age was calretinin positive neuron loss from the visual association laterodorsal thalamic nucleus and lateral posterior thalamic nucleus, (Figs. 1B and 10B), the reticular thalamic nucleus, zona incerta, and superficial layers of the superior colliculus (Fig. 10A) where cells receive direct input from small γ -type retinal ganglion cells (Hong et al., 2002). It has been suggested that collicular neuropathology may be a direct consequence of the independently developing retinal neuropathology (Graydon and Jolly, 1984; Mayhew et al., 1985). However ganglion cell inactivity due to monocular enucleation resulted in increased numbers of collicular calretinin positive neurons (Hong et al., 2002) whereas a numerical decline was observed in the superior and inferior colliculi, suggesting that neuron loss in this visual relay is independent of neuropathogenesis in the retina.

The CLN6 mutation also had contrasting effects on the soma area distributions of neurons in the parieto-occipital cortex (Fig. 4). Parvalbumin containing interneurons showed a hypertrophic phenotype whereas layer II and III pyramidal neuron somata tended to be smaller than their counterparts in age-matched controls. A similar phenotype of small cell bodies and retracted dendrites in layer II and III pyramidal neurons, also associated with thinning of the neocortex, has been induced by a gene targeting deletion of brain derived neurotrophic factor (BDNF) or its receptor tyrosine kinase B (TrkB) in post-natal mouse forebrains (Gorski et al., 2003; Xu et al., 2000). Neurotrophic factors, and BDNF signalling through TrkB receptor in particular, mediate aspects of synaptic plasticity instrumental in differentiating between more and less active synapses during the developmental pruning of excessive dendritic arbours (Katz and Shatz, 1996; Murer et al., 2001; Thoenen, 1995). It may be that experience-dependent modulation and protection of active synapses is disturbed during the post-natal development of CLN6 affected sheep brain.

Regional events and glial activation in NCL pathogenesis

It is intriguing that the regional distribution of the prominent early astrocytosis and microglial activation in affected sheep (Oswald et al., 2005; Kay et al., 2006) accurately predicts interneuron and pyramidal cell loss several months later. In addition to neuronal dysfunction or loss, there is mounting evidence that astrocyte dysfunction plays a central role in seizure generation and that astrocytes directly affect neuronal excitability (Tian et al., 2005; Volterra and Meldolesi, 2005).

The onset of glial activation during the perinatal development, progressive thinning of the cerebral cortex (Oswald et al., 2005; Kay et al., 2006), and the neuropathological involvement of pyramidal neuron and basket cell networks in visual and somatosensory cortical regions allow for the functional propagation of experience dependent plasticity to be primarily compromised in affected sheep. If cortical thinning during early postnatal development is linked to a reduction of dendritic trees of pyramidal cells then fewer cortical connections are formed and maintained. Microglial activation and neuropathology is first evident in layers II and III of all neocortical regions in affected sheep brains (Oswald et al., 2005). Neurons in these layers generally form ascending connections with other cortical regions (Felleman and Van Essen, 1991). Propagation of activity in the horizontal direction may be mediated via divergent translayer projections that terminate preferentially in layers II and III, or via intralayer projections that are most prominent in these layers (Gilbert, 1992; Thomson and Bannister, 2003). Since primary sensory cortical regions are affected more than higher associational cortical regions, higher brain centres receive less input and the overall cortical network is less active than normal. Neuronal responsiveness in states of arousal is enhanced by synaptic noise generated by an apparent randomness in cortical neuron activity (Destexhe and Marder, 2004). Thus reduced synaptic connectivity and associated neuronal activity may form a self-perpetuating mechanism leading to a general slowing of rhythmic brain activity.

In this scenario, the activation of astrocytes and microglia during early stages of brain pathology would represent a direct response to neuronal dysfunction, perhaps augmented by an intrinsic defect of the endosomal-lysosomal system. Astrocytes are increasingly recognised to play an active role in neuronal information processing and directly modulate neuronal activity (Seifert et al., 2006; Volterra and Meldolesi, 2005). As such, a functional defect in astrocytes may be a causative agent of pathological changes within neurons, but the regional differences in neuropathology are hard to rationalise by this mechanism. Neuropathological variations between cortical regions are explained better by functional differences in synaptic plasticity, demonstrated to occur between the primary motor and somatosensory cortices (Castro-Alamancos et al., 1995).

This study highlights the markedly different extents of interneuron loss between cortical and subcortical structures, emphasizing the value of studying the relative staging of pathological changes. Previous attempts to explain selective neuron vulnerability in the NCLs have focussed upon cellular identity (Cooper, 2003; Mitchison et al., 2004), however, our data of the close correlation between the regional distribution of astrogliosis, microglial activation and subsequent neuron loss in CLN6 sheep (Oswald et al., 2005; Kay et al., 2006; this study), emphasize cellular location and connectivity as more significant determinants of cell survival. Compared to mouse models, the relatively complex cortical mantle of sheep is ideally suited to revealing the pronounced regional hierarchy which underlies NCL pathogenesis. The selective involvements of functionally distinct CNS regions are more readily apparent, and the relative representation of cortical and subcortical structures more closely reflects the organization of the human CNS. In this respect, sheep models of NCL provide an invaluable resource for understanding the complex sequence of events during pathogenesis. Unravelling the underlying molecular mechanisms responsible for these events will significantly advance our understanding of their pathogenesis, important in deriving appropriate therapies, and may also enhance our understanding in cortical brain network function.

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C Calbindin

Level 2

Control **Affected**

Level 4

Fig. 1.

Immunohistochemical staining for interneuron phenotypes. Sagittal sections through the cerebral hemispheres of a year old adult control sheep and an affected sheep with advanced disease, aged 19 months, stained for parvalbumin (A), calretinin (B) and calbindin (C). Scale bar, 1 cm. A dramatic reduction in cortical immunoreactivity for parvalbumin in affected sheep contrasts with the relative preservation of subcortical staining (A), localised increases in staining for calretinin (B), and preservation of calbindin immunoreactivity (C). Note the heavy subcortical staining of both affected and control brains, particularly for calbindin.

Fig. 2.

Differential effects upon interneuron phenotypes. Immunostaining of the parietal cortex from a year old control sheep and affected sheep aged 6 and 19 months for parvalbumin (A), calretinin (B), calbindin (C) and somatostatin (D). Horizontal lines indicate boundaries between layers and between the cerebral cortex and white matter. Scale bar, 100 μ m. Examples of parvalbumin positive cells with pyramidal morphologies are marked with arrows in control panel (A). Loss of parvalbumin positive interneurons from affected sheep, evident at 6 months, becomes pronounced with age (A). In contrast, calretinin positive interneurons are relatively preserved (B), and calretinin immunoreactivity increased in the neuropil in a band mainly in layer I and Cajal-Retzius like cells of layers I and II. Calbindin immunoreactivity follows a similar pattern as in B (C). Loss of somatostatin positive interneurons from affected brain is apparent at 19 months (D).

Parvalbumin

A Parietal cortex

B Occipital cortex

Fig. 3.

Loss of parvalbumin positive interneurons from the cortex of affected sheep. Scale bar, 100 μ m. Immunostaining reveals the normal development of parvalbumin positive interneurons of the parietal cortex of 12 day old affected sheep (A), whereas there is a profound reduction in the number of parvalbumin positive neurons and neuropil immunoreactivity in layers II-III of the occipital cortex of an affected sheep at 19 months (B). Arrows mark the faintly visible axon terminal basket-like cell body outlines in affected sheep.

Fig. 4.

Cell size distributions of neuron subtypes. The size distributions of cell bodies in control and affected sheep parieto-occipital cortex at 4, 6, 12 and 19 months sorted into bins increasing in area by $50\mu\text{m}^2$. Each column represents the proportion of cells in each bin expressed as a percentage of the total number of cells, read from the ordinate. A higher proportion layer III pyramidal neurons staining with NeuroTrace late in disease are smaller (A), whereas the proportion of larger neurons immunoreactive for parvalbumin increases from 6 months (B). Larger cells disappear from the calretinin (C), and calbindin (D), positive populations while the size distributions of affected cells positive for somatostatin (E), and for neuropeptide Y (F), do not change.

Fig. 5.

Western blotting of calretinin in different regions of 6 month old control and affected sheep brains relative to β III-tubulin (A), and in 6 and 20 month old sheep normalised to β III-tubulin and expressed as ratios (B). Samples from an affected and a control sheep of each age were analysed in triplicate. The control bar is the mean of both ages, and the open circles and filled triangles the respective values for the 6 and 20 month old brains. Note the region specific increase in calretinin in affected visual cortex, motor cortex and hippocampus at 6 months which then becomes less evident at 19 months.

Entorhinal cortex

Control

Affected 19 months

A Calretinin

B Calbindin

Fig. 6.

Calretinin and calbindin immunoreactivities in the entorhinal cortex of severely affected sheep compared to a 6 month old control. Horizontal lines indicate boundaries between layers and between the cerebral cortex and white matter. Scale bars, 100 μ m. Immunohistochemical staining for calretinin (A), reveals the marked compression of principal cell layers in the affected entorhinal cortex at 19 months and the pronounced loss of calretinin positive interneurons, most notably from layer III. In contrast calretinin immunoreactivity becomes more intense in the neuropil of deeper layers of the affected entorhinal cortex. Immunohistochemical staining for calbindin (B) also reveals the pronounced loss of calbindin

positive interneurons from the severely affected entorhinal cortex, and increased immunoreactivity for calbindin in layer I.

A Nissl **B Parvalbumin, dentate gyrus** **Fig. 7.**

Changes in hippocampal interneurons. Nissl stained hippocampuses from a year old adult control and a severely affected 19 month old sheep (A). Boundaries between CA1, CA2, and CA3 and the subiculum are marked by arrowheads. Scale bar, 1 mm. Note the overall atrophy of the hippocampus and the pronounced loss of pyramidal neurons from the CA1 subfield. Changes in parvalbumin immunoreactivity within the hippocampal dentate gyrus (B). Scale bar, 100 μ m. The polymorphic layer (pm), granular layer (g) and molecular layer (m) are marked. Reduced staining of parvalbumin positive neurons and their processes within the neuropil of the polymorphic layer (pm) evident from 6 months decreases with age, very few neurons remaining in severely affected sheep.

CA1Control
12 monthsAffected
6 monthsAffected
12 monthsAffected
19 months**A Parvalbumin** **B Calbindin** **C Somatostatin**Control
12 monthsAffected
19 months **D Neuropeptide Y**Control
12 monthsAffected
19 months **Fig. 8.**

Changes in hippocampal CA1 interneurons shown by immunohistochemical staining for parvalbumin (A), calbindin (B), somatostatin (C), and neuropeptide Y (D). Scale bar, 100 μ m. The alveus (al), stratum oriens (o), stratum pyramidale (p), stratum radiatum (r), stratum lacunosum moleculare (lm) of the hippocampus, and the hippocampal fissure (hf), molecular layer (m), granular layer (g), polymorphic layer (pm) and hilus (h) of the dentate gyrus are marked. Loss of parvalbumin immunoreactive interneurons (A) is already evident at 6 months. Calbindin immunoreactivity (B) fluctuates during disease progression. It is reduced in the neuropil adjacent to the dentate gyrus of affected sheep at 12 months, is much more intense in dentate granule neurons and in the stratum oriens (o) adjacent to CA1 at this age, but then

becomes markedly reduced there at 19 months. Somatostatin immunoreactivity (C) is relatively preserved in severely affected sheep, although these neurons appear to be markedly hypertrophied. Neuropeptide Y immunoreactivity (D) is generally reduced in CA1 and the dentate gyrus of severely affected sheep, except for a band of increased neuropil staining in the stratum lacunosum moleculare (lm).

CA3

A Parvalbumin

B Calbindin

C Somatostatin

D Neuropeptide Y

Fig. 9.

Changes in hippocampal CA3 interneurons shown by immunohistochemical staining for parvalbumin (A), calbindin (B), somatostatin (C), and NPY (D). Scale bar, 100 μ m. The stratum oriens (o), stratum pyramidale (p), stratum lucidum (l) and stratum radiatum (r) are marked. Parvalbumin positive interneurons in CA3 (A) are progressively lost in affected sheep, few persisting at 19 months. Fewer calbindin stained neurons (B) are evident in CA3 of both control and affected sheep compared to CA1. A marked increase in calbindin positive fibres and terminals in the CA3 stratum lucidum (l) of affected sheep at 6 months declines to control intensity by severe disease. Somatostatin positive interneurons (C) were well preserved in severely affected sheep, but displayed distended cell soma. CA3 neuropeptide Y immunoreactivity (D) is markedly reduced.

Fig. 10.

Calretinin in subcortical brain structures of affected sheep. Scale bar, 100 μ m. Loss of calretinin positive interneurons from the superior colliculus of the 19 month old affected brain is pronounced (A) but calretinin positive neurites are preserved. The number of calretinin stained neurons in the severely affected lateral posterior thalamic nucleus is markedly reduced (B) but a subset of larger hypertrophied neurons stain intensely. Calretinin immunoreactivity is relatively well preserved within neurons of the substantia nigra pars compacta of affected sheep (C), but fewer dendrites and fibres are evident.