Progressive thalamocortical neuron loss in Cln5 deficient mice: Distinct effects in Finnish variant late infantile NCL

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Finnish variant LINCL (vLNCLFin) is the result of mutations in the CLN5 gene. To gain insights into the pathological staging of this fatal pediatric disorder, we have undertaken a stereological analysis of the CNS of Cln5 deficient mice (Cln5−/−) at different stages of disease progression. Consistent with human vLNCLFin, these Cln5−/− mice displayed a relatively late onset regional atrophy and generalized cortical thinning and synaptic pathology, preceded by early and localized glial responses within the thalamocortical system. However, in marked contrast to other forms of NCL, neuron loss in Cln5−/− mice began in the cortex and only subsequently occurred within thalamic relay nuclei. Nevertheless, as in other NCL mouse models, this progressive thalamocortical neuron loss was still most pronounced within the visual system. These data provide unexpected evidence for a distinctive sequence of neuron loss in the thalamocortical system of Cln5−/− mice, diametrically opposed to that seen in other forms of NCL.

Introduction

The neuronal ceroid lipofuscinoses (NCLs) are collectively the most common group of inherited pediatric neurodegenerative diseases. Their clinical symptoms include visual impairment, seizures, progressive psychomotor retardation and premature death (Santavuori, 1988). The NCLs are pathologically defined by a dramatic loss of CNS neurons, but with very few obvious effects outside the brain. All forms of NCL exhibit the characteristic accumulation of lysosomal autofluorescent lipopigments that display subtype specific ultrastructure (Mole et al., 2005). The NCLs are classified into ten different subtypes (CLN1–CLN10) on the basis of age of onset, clinicopathological features and genetic linkage, with eight genes identified to date (Stintola et al., 2006, 2007).

The Finnish variant form of late infantile neuronal ceroid lipofuscinosis (vLNCLFin, CLN5) is caused by mutations in the CLN5 gene, originally reported as encoding both soluble and membrane-bound forms of a lysosomal protein (Isosomppi et al., 2002; Vesa et al., 2002). vLNCLFin typically appears between 4 and 7 years of age, with the first symptoms usually being motor clumsiness followed by progressive visual failure and blindness, motor and mental deterioration, myoclonia and seizures culminating in an early death between 14 and 36 years (Santavuori et al., 1993; Santavuori et al., 1982). Although the function of the CLN5 protein has remained elusive, the consensus view is that its predominant form is a soluble lysosomal protein (Sleat et al., 2005).

Several animal models exist for different forms of NCLs and they provide excellent tools for thorough characterization of temporal and spatial pathological cascades (Cooper, 2003; Cooper et al., 2006). Mouse models of NCL share several common pathological similarities, including selective loss of GABAergic interneuron subpopulations, cortical and thalamic atrophy and pronounced early gliosis (reviewed in Mitchison et al., 2004; Cooper et al., 2006). More detailed pathological analyses of mouse models of congenital, infantile and juvenile forms of NCL, have all highlighted the thalamocortical pathways in pathogenesis with an early loss of thalamic relay neurons before the onset of neuron loss within the corresponding cortical region (Pontikis et al., 2005; Weimer et al., 2006; Kielar et al., 2007; Partanen et al., 2008).

Consistent with the milder NCL-phenotype of human vLNCLFin patients, Cln5−/− mice exhibit rather mild clinical symptoms (Kopra et al., 2004). These mice show widespread CNS accumulation of autofluorescent material, progressive visual failure and effects upon GABAergic interneuron survival (Kopra et al., 2004). Gene expression profiling of Cln5−/− mice revealed changes in the CNS expression levels for genes related to phosphorylation, cell adhesion, inflammation and myelin integrity (Kopra et al., 2004; von Schantz et al., 2008). Nevertheless, no detailed information is available about the sequence of neuropathological changes in these Cln5−/− mice. Since the
thalamocortical system displays a series of localized neurodegenerative and reactive changes in other models of NCL (Pontikis et al., 2005; Weimer et al., 2006; Kieler et al., 2007; Partanen et al., 2008), we focused our analysis on these pathways. Although Cln5 deficient mice share many similar features with other mouse models of NCL, our findings reveal that the sequence of neuron loss is unexpectedly different in Cln5/−/− mice, starting in the cortex and only subsequently becoming apparent in the thalamus.

Materials and methods

Animals

Homozygous mutant Cln5/−/− mice were generated on a mixed C57BL/6Jx129SvEv background as described previously (Kopra et al., 2004; Jalanko et al., 2005) and subsequently backcrossed for three generations with C57BL/6 controls to produce the mice used in this study. To provide a direct comparison with another NCL mouse model raised on the same strain background and housed in the same animal facility, we also collected brain tissue from male Cln1/−/− (Ppt1+/−/−) mice (Jalanko et al., 2005) at 1 and 4 months of age. We utilized systematically sampled brain tissue from 1, 4 and 12 month old male mice for all experiments with separate wild-type littermate utilized systematically sampled brain tissue from 1, 4 and 12 month old male mice for all experiments with separate wild-type littermate mice used as controls for each mutant strain. The genotypes of all mice were determined by polymerase chain reaction of DNA from tail biopsies (Kopra et al., 2004; Jalanko et al., 2005). All animal experiments were conducted in accordance with international standards on animal welfare and with approved animal policies of the National Public Health Institute, Helsinki, with adequate measures taken to minimize pain or discomfort.

Histological processing

For histological analysis, Cln5−/−, Cln1−/− and age-matched control mice (n = 3 per genotype and age) were euthanized at 1 month and 4 months of age (both strains of mutant mice) and 12 months of age (Cln5−/− only) in a rising concentration of carbon dioxide, their brains were removed and bisected along the midline. One half of the bisected brain was frozen in liquid nitrogen and stored in −80 °C and the other hemisphere was fixed in 4% paraformaldehyde in 0.2 M phosphate buffer for at least one week before cryoprotection in 30% sucrose in 50 mM Tris buffered saline (TBS) containing 0.05% Sodium Azide. From these cryoprotected brains 40 μm frozen coronal sections were cut through the cortical mantle (Leitz 1321 freezing microtome, Leica Microsystems, Welwyn Garden City, UK), while the cerebellum was cut sagittally. As described previously sections were collected, one per well, into 96 well plates containing a cryoprotectant solution (Bible et al., 2004; Kieler et al., 2007), and stored at −80 °C before histological processing. To provide direct visualization of neuronal morphology each adjacent section was slide mounted and Nissl stained as described previously (Bible et al., 2004). All histological processing and subsequent analyses were performed with no prior knowledge of genotype or treatment group.

Regional volume measurements

To examine regional volume, unbiased Cavalieri estimates of the cortex, hippocampus, striatum, thalamus, hypothalamus and cerebellum were performed with no prior knowledge of genotype or treatment group. Individual laminar thicknesses were also measured in M1, S1BF and V1 using the same three consecutive Nissl-stained sections. The thickness of each individual lamina was measured via ten perpendicular lines using an ×10 objective. Results were expressed as mean laminar thickness in μm per region for Cln5−/− mice and controls.

Counts of neuronal number

To examine neuronal survival and volume within individual thalamic nuclei and their target cortical regions we used StereoInvestigator software to obtain unbiased optical fractionator estimates of the number of Nissl stained neurons in each structure, with the boundaries of each nucleus as defined by Paxinos and Franklin (2001). These measures were performed in the ventral posterior nucleus (VPM/VPL), dorsal lateral geniculate nucleus (LGNd), which project to S1BF and V1, respectively. In these cortical regions we obtained optical fractionator estimates of the number of Nissl stained granule neurons in lamina IV that receive thalamic innervation, lamina VI neurons that supply feedback to the thalamus, and lamina V projection neurons. These measures were performed exactly as described previously (Bible et al., 2004; Kieler et al., 2007), with a random starting section chosen, followed by every sixth Nissl stained section. All counts were carried out using a ×100 oil objective (NA 1.4), with only neurons with a clearly identifiable nucleolus counted.

Immunohistochemistry

To assess the extent of glial activation, adjacent one in six series of free floating 40 μm frozen sections were immunohistochemically stained, as described previously (Bible et al., 2004), for detection of astrocytic (GFAP, DAKO, Cambridge, UK, 1:5000) and microglial (F4/80, Serotec, Oxford, UK, 1:100) markers. Briefly, sections were incubated for 15 min in 1% hydrogen peroxide in TBS to quench endogenous peroxidase activity, rinsed in TBS and incubated for 40 min in TBS/0.3% Triton X-100 (TBS-T) containing 15% normal serum to block non-specific binding of immunoglobulins. Sections were incubated overnight at 4 °C in primary antiserum diluted with 10% normal serum in TBS-T, and subsequently rinsed in TBS and incubated for 2 h in biotinylated secondary antiserum diluted with 10% normal serum in TBS-T. Following rinsing in TBS, sections were incubated for 2 h in Vectorstain avidin–biotin–peroxidase complex (Elite ABC kit, Vector Laboratories, Peterborough, UK) diluted with 10% normal serum in TBS-T and rinsed again in TBS. Immunoreactivity was visualized by a standard DAB reaction (Sigma, Dorset, UK), and sections were transferred to excess ice-cold TBS, mounted onto gelatine-chrome alum coated Superfrost microscope slides (VWR, Dorset, UK), air-dried overnight and passed through a graded series of alcohol before clearing in xylene and coverslipping with DPX mounting media (VWR).

Quantitative analysis of glial phenotype

The optical density of GFAP and F4/80 immunoreactivity was assessed using a semi-automated thresholding image analysis, as
previously described (von Eitzen et al., 1998; Bible et al., 2004; Kielar et al., 2007), with each marker analyzed blind with respect to genotype or age. Briefly, 40 nonoverlapping images, on triplicate sections, were captured through each selected brain region (GFAP and F4/80; VPM/VPL and S1BF). The optimal segmentation of immunoreactive profiles was determined using Optimas image analysis software (Media Cybernetics, Silver Springs, MD) using a previously described semi-automated thresholding method based on the optical density of the reaction product (von Eitzen et al., 1998; Bible et al., 2004; Kielar et al., 2007). Macros were recorded to transfer the data to a spreadsheet for subsequent statistical analysis. Data were plotted graphically as the mean percentage area of immunoreactivity per field ± SEM for each region.

Statistical analysis

The statistical significance of differences between genotypes of all quantitative data was assessed using a one-way ANOVA (SPSS 11.5 software, SPSS Inc, Chicago, IL), with statistical significance considered at p < 0.05. The mean co-efficient of error (CE) for all individual optical fractionator and Cavalieri estimates was calculated according to the method of Gundersen and Jensen (1987) and was less than 0.08 in all these analyses.

Results

Regional atrophy and cortical thinning in Cln5−/− mice

Apart from an early and progressive visual failure Chn5 null mutant mice (Cln5−/−) display a relatively late onset disease phenotype (Kopra et al., 2004). To survey effects upon the CNS in symptomatic mutant mice, we carried out a stereological survey of regional volume in Nissl-stained sections of Cln5−/− mice and littermate controls at 12 months of age. Cavalieri estimates of regional volume revealed significant atrophy of the cortex, hippocampus, striatum and thalamus of these aged Cln5−/− mice, but no significant effects upon the volume of the hypothalamus and cerebellum (Fig. 1A).

Cortical atrophy is a consistent feature of all forms of human and murine NCL (Cooper et al., 2006), but does not usually occur equally across the cortical mantle (e.g. Bible et al., 2004; Kielar et al., 2007). To
investigate whether similar regional effects were apparent in \textit{Cln5} deficient mice, we made thickness measurements in Nissl stained sections through the primary motor cortex (M1), somatosensory barrelefield cortex (S1BF), primary visual cortex (V1), and lateral entorhinal cortex (LEnt), as representative cortical regions that serve different functions. Compared with littermate controls, 12 month old \textit{Cln5} \textsuperscript{−/−} mice exhibited significant thinning in all cortical regions (Fig. 1B), suggesting widespread rather than regionally specific effects upon the cortical mantle.

To determine whether the reduced thickness of these cortical regions was due to laminar specific events, we made a series of individual laminar thickness measurements in the same Nissl stained sections (Figs. 2A–D). These measurements revealed more pronounced effects upon deeper cortical laminae in \textit{Cln5} deficient mice, with significant thinning of lamina V in all four cortical regions (Figs. 2A–D), and significant thinning of laminae IV and VI in S1BF, V1 and LEnt (Figs. 2B–D). In contrast, more superficial laminae exhibited a series of complex series of changes in thickness in 12 month old \textit{Cln5} \textsuperscript{−/−} mice. For example, M1, S1BF and V1 of \textit{Cln5} mutant mice displayed a significantly increased thickness of laminae I and II/III (Figs. 2B–D), and a significant reduction in thickness of lamina I, and a significantly increased thickness of lamina III within LEnt (Fig. 2D).

**Synaptic pathology in \textit{Cln5} deficient mice**

Mouse models of congenital, infantile and juvenile NCL display a range of synaptic pathologies (Partanen et al., 2008; Kim et al., 2008; Kielar et al., unpublished observations), which are most pronounced within the somatosensory and visual thalamocortical pathways. To determine whether severely affected \textit{Cln5} \textsuperscript{−/−} mice display a similar phenotype, we stained sections from these mice for a range of pre-synaptic markers, including the synaptic vesicle protein synaptobrevin, the pre-synaptic membrane protein synaptophysin and the SNARE complex protein SNAP25. We concentrated our analysis upon the ventral posterior thalamic nucleus (VPM/VPL) and the dorsal lateral geniculate nucleus (LGNd), which relay sensory information to the primary somatosensory (S1BF) and primary visual (V1) cortical subfields, respectively and display pronounced synaptic pathology in \textit{Cathepsin D} deficient mice (Partanen et al., 2008). Immunohistochemical staining for these proteins revealed pronounced changes in the intensity and distribution of staining for these markers in the thalamocortical system of 12 month old \textit{Cln5} \textsuperscript{−/−} mice. In these severely affected mutant mice, SNAP25 and synaptophysin immunoreactivity were markedly reduced in both VPM/VPL and LGNd of 12 month old \textit{Cln5} \textsuperscript{−/−} mice, whereas VAMP2 immunoreactivity appeared upregulated and around the persisting neurons in these thalamic nuclei (Figs. 3A and B). Changes in staining for synaptic markers were also evident in the corresponding cortical regions of these severely affected mutant mice, with markedly decreased staining for synaptophysin and redistribution of VAMP2 immunoreactivity in both S1BF and V1. However, in marked contrast to the thalamus, SNAP25 immunoreactivity was relatively preserved in the cortex of 12 month old \textit{Cln5} \textsuperscript{−/−} mice and displayed similar staining intensity in S1BF and V1 of mice of both genotypes.
genotypes (Fig. 3C). Taken together these data provide the first evidence for synaptic pathology in vLINCLFin, and suggest that this may be different in nature in the thalamus and cortex of Cln5 deficient mice.

Mouse models of NCL consistently display localized and progressive glial activation, which typically precedes neuron loss and is
associated with synaptic pathology (Cooper et al., 2006; Partanen et al., 2008), with the thalamocortical system emerging as a particular focus for these events (e.g., Bible et al., 2004, Pontikis et al., 2005, Kielar et al., 2007; Partanen et al., 2008). To determine whether Cln5 deficient mice share this phenotype, we examined adjacent series of sections from control and mutant mice between 1 and 12 months of age stained with the astrocytic marker GFAP (Fig. 4) or the microglial marker F4/80 (Fig. 6). As representative nuclei that display pronounced reactive changes in mouse models of congenital, infantile and juvenile NCL (Pontikis et al., 2005; Weimer et al., 2006; Kielar et al., 2007; Partanen et al., 2008), we again concentrated our analysis upon the thalamic nuclei VPM/VPL, and LGNd, together with S1BF and V1, the cortical regions to which these nuclei relay sensory information.

Astrocytosis

In both S1BF and V1 of control mice of all ages only few GFAP-immunoreactive astrocytes were present (Figs. 4A and C), predominantly in laminae I and VI, with additional scattered positive cells in lamina V. At 1 month of age the distribution of GFAP immunoreactivity in S1BF (Fig. 4A) and V1 (Fig. 4C) was similar in Cln5−/− mice. However, in these mutant mice at 4 months of age the number and density of GFAP stained protoplasmic astrocytes was increased within all laminae of S1BF (Fig. 4A) and V1 (Fig. 4C), with the exception of lamina IV, which contained very few positively stained astrocytes in either cortical region. At 12 months of age both cortical regions exhibited widespread astrocytosis in Cln5 deficient mice, with intensely GFAP-immunoreactive astrocytes present in all laminae (Figs. 4A and C).

In the thalamus, Cln5−/− mice displayed a progressive astrocytosis that became more pronounced with increased age (Figs. 4B and D). Within VPM/VPL, numerous scattered GFAP-positive astrocytes were already evident in mutant mice at 4 months of age (Fig. 4B), but were virtually absent in age-matched controls. By 12 months of age intense GFAP immunoreactivity was now evident throughout VPM/VPL, spreading into adjacent nuclei (Fig. 4B). In contrast, there was no discernable difference in the distribution of GFAP immunoreactivity in LGNd (Fig. 4D) or MGN (data not shown) of mutant and control mice, until 12 months of age when both of these nuclei were completely filled with intensely immunoreactive astrocytes in Cln5 deficient mice.

Quantitative thresholding image analysis confirmed the significantly increased astrocytosis within both VPM/VPL (Fig. 5A) and S1BF (Fig. 5B) of Cln5 deficient mice with increased age. Consistent with

Fig. 4. Progressive astrocytosis in the thalamocortical system of Cln5 deficient mice. (A–D) Immunohistochemical staining for glial fibrillary associated protein (GFAP) reveals the pronounced upregulation of this marker of astrocytosis with increased age in the somatosensory barrel field cortex (S1BF, A) primary visual cortex (V1, C) of Cln5−/− mice compared to age matched controls (+/+). (A, C) At one month of age only few scattered GFAP immunoreactive astrocytes were evident mostly in deeper laminae of both S1BF and V1 in control mice, with similar staining evident in Cln5−/− mice. In these mutant mice at 4 months of age GFAP immunoreactivity became more pronounced in both deeper laminae (IV–VI) and superficial laminae (I–III) of both cortical regions, becoming markedly more intense and spreading to involve all laminae at 12 months of age. Laminar boundaries are indicated by roman numerals. (B, D) A similar progressive increase in the distribution and intensity of GFAP immunoreactivity was also evident in the ventral posterior (VPM/VPL, B) and dorsal lateral geniculate (LGNd, D) thalamic nucleus, which project to S1BF and V1, respectively. At 1 month of age GFAP immunoreactivity appeared similar in control and Cln5−/− mice, but numerous scattered GFAP-positive astrocytes were evident in VPM/VPL at 4 months of age. In contrast LGNd contained few GFAP immunoreactive astrocytes until 12 months of age when intense GFAP staining delineated both VPM/VPL and LGNd. The boundaries of these nuclei are indicated by white dashed lines. Scale bar = 300 μm.
our histological observations this increased astrocytosis was of greater magnitude in the thalamus of mutant mice at each age examined. Although of a small magnitude, the upregulation of GFAP immunoreactivity was already significant in the thalamus and cortex of 1 month old Cln5 deficient mice (Fig. 5), and became more pronounced with increased age, especially within the thalamus.

Microglial activation

Before 12 months of age, there were no discernable differences between genotypes in the extent and distribution of F4/80 immunoreactivity, but staining for this marker revealed pronounced and localized microglial activation in 12 month old Cln5 deficient mice (Fig. 6). At all ages control mice displayed widespread distribution of ramified microglia in the S1BF (Fig. 6A) and V1 (Fig. 6C), revealed by pale F4/80 immunoreactivity. In contrast, a higher level of microglial activation was seen in both S1BF and V1 of Cln5 deficient mice, with intensely F4/80 immunoreactive microglia predominantly within deeper laminae, with occasional darkly stained microglia in more superficial laminae (Figs. 6A and C). Compared to the palely stained and highly ramified microglia evident in the cortex of control mice (Figs. 6A and C), microglia in Cln5 deficient mice displayed a larger more intensely stained soma with numerous short thickened processes. Differences between genotypes in the relative intensity of F4/80 immunoreactivity and microglial morphology were more prominent in subcortical structures (Figs. 6C and D). 12 month old Cln5 deficient mice displayed localized microglial activation within individual thalamic nuclei (Figs. 6B and D), in contrast to widespread distribution of palely stained microglia in age-matched littermate controls. Morphologically, many of the intensely stained microglia in mutant mice displayed an enlarged soma and short thickened processes typical of an amoeboid or brain macrophage like appearance (Fig. 6D), versus the less intensely stained microglia with thin ramified processes present in age-matched controls. This dramatic upregulation of F4/80 immunoreactivity was confirmed by thresholding image analysis revealing significantly increased microglial activation in the both S1BF and VPM/VPL in 12 month old Cln5 deficient mice (Fig. 6E).

Late onset thalamic relay neuron loss in Cln5 deficient mice

In other forms of NCL the thalamic nuclei that exhibit localized astrocytosis, subsequently display neuron loss several months before neurons in their corresponding cortical target regions (Kieler et al., 2007). To investigate whether Cln5 deficient mice displayed a similar relationship between thalamic and cortical neuron loss we used unbiased stereology to determine the extent of neuronal loss in Nissl stained sections within three thalamic nuclei (Fig. 7) that relay different sensory modalities to the cortex (somatosensory, ventral posterior thalamic nucleus VPM/VPL; visual, dorsal lateral geniculate nucleus LGNd; auditory, medial geniculate nucleus MGN). Optical fractionator estimates of neuron number in Cln5 deficient mice revealed selective effects upon thalamic relay neuron survival that occurred relatively late in disease progression. There was a significant loss of VPM/VPL and LGNd neurons in Cln5 deficient mice that only became evident at 12 months of age, but no significant loss of MGN neurons even in these aged mutant mice (Fig. 7A).

Early onset cortical neuron loss in Cln5 deficient mice

Since these changes in thalamic neuron number only became evident once cortical atrophy was apparent, we next investigated the relative timing of cortical neuron loss in Cln5 mutant mice and age-matched controls at 4 and 12 months of age. We focused our analysis upon S1BF and V1, the cortical target regions of the two thalamic relay nuclei (VPM/VPL and LGNd respectively), which displayed significant neuron loss at 12 months. To do this we obtained optical fractionator estimates in mice of both genotypes of the number of Nissl stained granule neurons in lamina IV granule neurons that receive thalamic innervation, lamina VI neurons that supply feedback to the thalamus, and lamina V projection neurons (Figs. 7B and C).

Surprisingly, this analysis revealed a relatively early and significant loss of cortical neurons in Cln5 deficient mice at 4 months of age, which became more widespread and involved more laminae with increased age. Although present in both cortical regions of Cln5 deficient mice, this neuron loss was more pronounced and occurred earlier within the visual system with significant effects upon the number of lamina IV granule neurons and lamina V projection neurons in V1 already evident at 4 months of age (Fig. 7C). In contrast, the somatosensory cortex was better preserved at this age with significant neuron loss only present in lamina V of S1BF at 4 months of age, with a significant loss of lamina IV neurons also observed in Cln5 mutant mice at 12 months of age (Fig. 7B).

This relatively early onset of cortical neuron loss in Cln5 mutant mice is in marked contrast to our findings in mouse models of other forms of NCL (Weimer et al., 2006; Kieler et al., 2007; Partanen et al., 2008). To provide a direct comparison to another NCL mouse model that was raised on the same mixed background and housed in the same animal house, we next surveyed thalamocortical relationship between thalamic and cortical neuron loss we used unbiased stereology to determine the extent of neuronal loss in Nissl stained sections within three thalamic nuclei (Fig. 7) that relay different sensory modalities to the cortex (somatosensory, ventral posterior thalamic nucleus VPM/VPL; visual, dorsal lateral geniculate nucleus LGNd; auditory, medial geniculate nucleus MGN). Optical fractionator estimates of neuron number in Cln5 deficient mice revealed selective effects upon thalamic relay neuron survival that occurred relatively late in disease progression. There was a significant loss of VPM/VPL and LGNd neurons in Cln5 deficient mice that only became evident at 12 months of age, but no significant loss of MGN neurons even in these aged mutant mice (Fig. 7A).
evident at 4 months of age, but with no effects upon cortical neuron survival in these mice at this age (Fig. 8). These data not only confirm our previous findings in another mouse model of INCL (Kielar et al., 2007), but emphasize the unexpected nature of our findings in Cln5 deficient mice, which display a completely reversed sequence of neuron loss occurring first within the cortex and only subsequently in the thalamus (Figs. 7 and 9).

**Discussion**

Characterization of NCL mouse models is revealing a wealth of new information about disease progression and this study provides the first detailed description of the sequence of pathological events in Cln5−/− mice. As in mouse models of other forms of NCL, the thalamocortical system of these Cln5 deficient mice displayed...
Fig. 7. Progressive loss of thalamic and cortical neurons in Cln5 deficient mice. (A) Histograms of unbiased optical fractionator estimates of the number of Nissl stained thalamic relay neurons in the ventral posterior (VPM/VPL), medial geniculate nucleus (MGN), and dorsal lateral geniculate nucleus (LGNd), of Cln5<sup>−/−</sup> mice and age-matched controls (+/+) at different stages of disease progression. The number of neurons in VPM/VPL and LGNd nuclei declined in Cln5<sup>−/−</sup> mice with increased age, with significant neuron loss evident in these nuclei in 12 month old mutant mice. In contrast no significant loss of MGN neurons was observed in Cln5<sup>−/−</sup> mice at either age. (B, C) Histograms of unbiased optical fractionator estimates of the number of Nissl stained lamina IV granule neurons, lamina V pyramidal neurons and lamina VI feedback neurons in the somatosensory cortex (S1BF; B) and primary visual cortex (V1, C) of Cln5<sup>−/−</sup> mice and age-matched controls (+/+) at different stages of disease progression. Both cortical regions displayed a progressive loss of cortical neurons in mutant mice, but this occurred earlier and in all laminae of V1. A significant loss of laminae IV and V neurons was already apparent in V1 of Cln5 deficient mice at 4 months of age, with additional significant neuron loss in lamina VI at 12 months of age. Neuron loss in S1BF progressed more slowly, but a significant loss of lamina V neurons was already evident in Cln5 deficient mice at 4 months of age, with an additional significant neuron loss in lamina IV of these mice at 12 months of age (*p<0.05; **p<0.01; ***p<0.001, ANOVA with post-hoc Bonferroni analysis).

Fig. 8. Early loss of thalamic relay neurons in Ppt1<sup>Δex4</sup> knock-in mice. Histograms of unbiased optical fractionator estimates of the number of Nissl stained lamina IV granule neurons and lamina V projection neurons in somatosensory barrel field (S1BF) cortex; the ventral posterior nucleus of the thalamus (VPM/VPL), which provides afferent input to S1BF; and visual relay neurons in the dorsal lateral geniculate thalamic nucleus (LGNd) of Ppt1<sup>Δex4</sup> mice and age-matched controls (+/+) at different stages of disease progression. No significant loss of cortical neurons in laminae IV or V of S1BF was evident in Ppt1<sup>Δex4</sup> mice at either 1 or 4 months of age. In marked contrast, consistent with the phenotype of Ppt1<sup>−/−</sup> mice (Kielar et al., 2007), a significant loss of both VPM/VPL and LGNd neurons was already observed at 4 months of age (*p<0.05; ***p<0.001, ANOVA with post-hoc Bonferroni analysis).
localized reactive changes and progressive neuron loss that became more pronounced with increased age. However, in marked contrast to all other forms of NCL characterized to date (Fig. 9), neuron loss in Cln5−/− mice began in the cortex and only subsequently occurred in the corresponding thalamic relay nuclei. These surprising data reveal that neuron loss in Cln5−/− mice progresses through the thalamocortical pathways in the opposite direction to that seen previously in all other forms of NCL.

Common themes in NCL pathogenesis?

The recent availability of mouse models for seven different forms of NCL has seen a series of studies describing the pathological phenotype of each disease model (reviewed by Cooper et al., 2006). Phenotypically these mice resemble the human disorder, displaying accumulation of autofluorescent storage material, profound astrocytosis and microglial activation, widespread neuron loss and brain atrophy (Cooper et al., 1999; Mitchison et al., 1999; Biddle et al., 2004; Kopra et al., 2004; Pontikis et al., 2004, 2005; Sleat et al., 2004; Tyynelä et al., 2004; Jalanko et al., 2005; Kielar et al., 2007; Partanen et al., 2008). Our data from 12 month old Cln5 deficient mice reveal a similar phenotype with widespread regional atrophy and generalized thinning of the cortex (Fig. 1), prominent astrocytosis and microglial activation that are most pronounced within the thalamocortical system (Figs. 4 and 6). Coming at the end of a late onset and slowly progressing NCL-like disorder, this phenotype closely resembles that of Chn3 mutant models of juvenile NCL (JNCL) (Mitchison et al., 1999; Cotman et al., 2002; Pontikis et al., 2004, 2005). This is in contrast to much earlier onset and rapidly progressing degenerative phenotypes of mouse models of infantile (Gupta et al., 2001; Jalanko et al., 2005; Kielar et al., 2007), and especially congenital NCL (Partanen et al., 2008), suggesting a correlation between disease severity in these models and the corresponding human condition. However, as our findings in Cln5 deficient mice highlight, although these mouse models display similar pathological endpoints (Mitchison et al., 2004; Cooper et al., 2006), these may be preceded by a sequence of neuron loss within thalamocortical pathways that progresses differently between forms of NCL. Indeed, it is only by systematically studying the staging of these disorders that such important differences can become apparent.

Despite the fundamental difference in their relative timing of cortical and thalamic neuron loss (Fig. 9), one consistent feature of NCL pathogenesis that is retained in Cln5−/− mice is the early targeting of the visual system. For example, visual relay neurons are

Fig. 9. Contrasting patterns of corticothalamic neuron loss in NCL mouse models. (A) Normal organization of thalamocortical pathways. Ascending sensory afferents terminate upon thalamic relay neurons, with one relay nucleus for information of each modality. These relay neurons project mainly to lamina IV granule neurons of the appropriate cortical region, with collateral innervation of lamina VI. In turn, the cortex provides feedback projections to the thalamus from lamina VI, and lamina V neurons project outside this system (Figs. 4 and 6). Coming at the end of a late onset and slowly progressing NCL-like disorder, this phenotype closely resembles that of Ppt1Δex4 knock-in mice on the same background (Jalanko et al., 2005 and Fig. 8), and housed in the same animal facility exhibit a phenotype remarkably similar to that of Ppt1−/− deficient mice on a congeneric background (Kielar et al., 2007). Indeed our findings from both INCL and JNCL mouse models reveals very little difference between the NCL phenotypes of these mice upon mixed strain or different congeneric mouse strains (Bible et al., 2004; Pontikis et al., 2004, 2005; Kielar et al., 2007; Cooper and Pearce, unpublished observations).

In contrast, neuron loss progresses in a completely opposite sequence in Cln5 deficient mice. As this study reveals, neuron loss is first evident in the cortex of Cln5−/− mice, with loss of thalamic relay neurons occurring later in disease progression. However, regardless of the pattern of cell loss, visual pathways are consistently affected first in all NCL mouse models.
lost before somatosensory relay neurons in Cln3 and Ppt1 null mutant mice (Weimer et al., 2006; Kielar et al., 2007), and in Ppt1\textsuperscript{fl/fl} mice. LGNd neurons are also lost early in disease progression (Fig. 8). Indeed, such early loss of visual relay neurons, suggests that visual failure is due to pathological effects on central visual pathways rather than solely retinal degeneration (Weimer et al., 2006). Changes in the expression of several genes that play important roles within the visual system are evident in Cln5 deficient mice (von Schantz et al., 2008). Moreover, the onset of neuron loss in the primary visual cortex in these Cln5 deficient mice (Fig. 7) coincides with the onset of visual failure in these mice (Kopra et al., 2004). Although the basis of visual dysfunction in these mice remains unclear, it will be important to define the relationship between neuron loss within the retina and more central components of the visual system in Cln5\textsuperscript{−/−} mice.

Although astrocytosis has long been considered a hallmark of neurodegenerative changes, it is now apparent that glial responses often precede neuron loss and may be indicative of neuronal dysfunction (Raivich et al., 1999). Indeed, the early glial responses that occur in multiple forms of NCL and other LSDs accurately predict where neuron loss subsequently occurs (Oswald et al., 2005; Kielar et al., 2007; reviewed by Castañeda et al., 2008). In this respect the presence of early and localized astrocytosis within the thalamus of Cln5\textsuperscript{−/−} mice (von Schantz et al., 2008) has implied that the same cellular pathways are affected differently between these regions (Fig. 3), hinting that dissimilar pathological events are happening in the thalamus and cortex of Cln5 deficient mice. It will be important to determine the relationship between these synaptic pathologies and the distinctive sequence of neuron that occurs in the thalamocortical system of these Cln5\textsuperscript{−/−} mice.

**Implications for therapy?**

The characteristic and unexpected pattern of neuron loss in Cln5 deficient mice highlights the need to determine the mechanisms that underlie neuron vulnerability in these disorders. However, regardless of the underlying events, the discovery of cortical rather than thalamic predominance in vLNICEFP pathogenesis has important implications for the targeting of therapies for this disorder. Indeed, if therapies are to be delivered to where and when they can be most effective, these data highlight the need to study the progressive pathological changes that lead up to the end stages of any disease. This is particularly relevant for vLNICEFP, since the CLN5/Cln5 gene product has now been identified as a soluble lysosomal glycoprotein that appears to be trafficked via the mannose-6-phosphate pathway (Holmberg et al., 2004; Sleat et al., 2005). This discovery implies that cross-correction of Cln5 deficient neurons is possible, paving the way for gene therapy approaches that will be most likely to succeed if targeted to where pathology is first apparent. In this respect our findings from Cln5\textsuperscript{−/−} mice are likely to be particularly informative and the recent confirmation of a large animal model of CLN5 deficiency (Fugier et al., 2008), provides an ideal opportunity to test methods for the effective delivery of appropriately targeted therapy in this profoundly disabling disorder.

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**References**


Kopra et al., 2004.
Cooper, J.D., 2003. Progress towards understanding the neurobiology of Batten disease