Thalamocortical neuron loss and localized astrocytosis in the Cln3\textsuperscript{-\textit{del}ex7/8} knock-in mouse model of Batten disease

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Juvenile neuronal ceroid lipofuscinosis (JNCL) is the result of mutations in the Cln3 gene. The Cln3 knock-in mouse (Cln3\textsuperscript{-\textit{del}ex7/8}) reproduces the most common Cln3 mutation and we have now characterized the CNS of these mice at 12 months of age. With the exception of the thalamus, Cln3\textsuperscript{-\textit{del}ex7/8} homozygotes displayed no significant regional atrophy, but a range of changes in individual laminar thickness that resulted in variable cortical thinning across subfields. Stereological analysis revealed a pronounced loss of neurons within individual laminae of somatosensory cortex of affected mice and the novel finding of a loss of sensory relay thalamic neurons. These affected mice also exhibited profound astrocytic reactions that were most pronounced in the neocortex and thalamus, but diminished in other brain regions. These data provide the first direct evidence for neurodegenerative and reactive changes in the thalamocortical system in JNCL and emphasize the localized nature of these events.

Keywords: Batten disease; Juvenile neuronal ceroid lipofuscinosis; JNCL; CLN3; Thalamocortical degeneration; Astrocytosis; Lysosomal storage disorder

Introduction

The neuronal ceroid lipofuscinoses (NCLs) are the most common group of inherited neurodegenerative disorders of childhood with a collective incidence of up to 1 in 12,500 live births (Cooper, 2003; Hofmann and Peltonen, 2001). These fatal lysosomal disorders are characterized by blindness, marked psychomotor deterioration and uncontrollable seizures (Hofmann and Peltonen, 2001; Gardiner, 2002). Juvenile NCL (JNCL) is the result of a mutation in the CLN3 gene, located in the p12.1 region of chromosome 16 (International Batten Disease Consortium, 1995). This most frequently occurring form of NCL has an age of onset between 4 and 10 years and typically results in premature death before the age of 30 (Gardiner, 2002).

The Cln3 protein is predicted to be a highly hydrophobic 438 amino acid protein, containing multiple transmembrane domains (Mao et al., 2003). Although very little is known about the normal function of Cln3, colocalization studies have shown this protein to be expressed in the lysosome, endosome and synaptosomes (Haskell et al., 2000; Jarvela et al., 1999). The most significant advances in understanding Cln3 function come from yeast that bear null mutations in BTN1p, the yeast ortholog of Cln3 (Pearce et al., 1999). The deletion of BTN1 results in a reduced vacuolar pH during early growth (Pearce et al., 1999), and influences the transport of basic amino acids into the vacuole (Kim et al., 2003), suggesting that BTN1p may play a role in pH homeostasis.

Detailed quantitative information about neuropathological changes in JNCL is limited with qualitative studies restricted to autopsy material (Braak and Goebel, 1978, 1979). The loss of cortical neuron populations in JNCL has been well documented (Braak and Goebel, 1978, 1979; Haltia, 2003), together with selective neuronal loss and glial activation within the hippocampal formation (Haltia et al., 2001; Tyynelä et al., 2004). Since human autopsy material is not widely available, Cln3 null mutant mice (Cln3\textsuperscript{-/-}) have been generated to enable the study of JNCL pathogenesis (Katz et al., 1999; Mitchison et al., 1999). These animals exhibit a JNCL-like phenotype and display widespread intracellular accumulation of autofluorescent pigment and a selective loss of GABAergic interneurons (Mitchison et al., 1999; Katz et al., 1999; Pontikis et al., 2004), together with an altered threshold for seizure generation (Kriscenski-Perry et al., 2002). A distinctive feature of Cln3\textsuperscript{-/-} mice is the early occurrence of reactive changes in both astrocytic and microglial...
cell populations, events which occur many months before widespread neuronal loss is evident (Pontikis et al., 2004). Cln3<sup>−/−</sup> mice and individuals with JNCL also raise autoantibodies to glutamic acid decarboxylase (GAD65) that inhibit the activity of this enzyme, resulting in elevated levels of glutamate (Chattopadhyay et al., 2002).

More recently, Cln3 knock-in mice (Cln3<sup>ex7/8</sup>) have been generated which accurately reproduce the 1.02-kb deletion in the Cln3 gene that is seen in over 85% of JNCL alleles (International Batten Disease Consortium, 1995), and also exhibit an NCL-like phenotype (Cotman et al., 2002). This phenotype may be more aggressive than that seen in Cln3<sup>−/−</sup> mice, with a progressive neurologic disease and premature death of homozygous Cln3<sup>ex7/8</sup> mice from 7 months onwards (Cotman et al., 2002).

To further characterize the CNS of homozygous Cln3<sup>ex7/8</sup> mice, we examined neurodegenerative and reactive phenotypes that are present in other mouse models of NCL, including Cln3<sup>−/−</sup> mice (Mitchison et al., 1999; Pontikis et al., 2004). These models display a range of effects upon cortical thinning and neuronal loss between sensory and motor cortex (Bible et al., 2004; Cooper et al., 1999; Mitchison et al., 1999; Pontikis et al., 2004). Our analysis of these regions in homozygous Cln3<sup>ex7/8</sup> mice also revealed different degrees of cortical thinning and selective effects upon cortical neuron number, together with significant loss of afferent thalamic neurons. These neuronal changes were accompanied by graded microglial activation and pronounced hypertrophy, but not hyperplasia, of astrocytes. These reactive changes also exhibited marked regional specificity, being most profound in the neocortex hyperplasia, of astrocytes. These reactive changes also exhibited marked regional specificity, being most profound in the neocortex.

Materials and methods

Animals

Homozygous Cln3<sup>ex7/8</sup> knock-in mice and control (+/+) mice were generated on a mixed 129Sv/Ev/CD1 strain background at the Charles River, MA, as described previously (Cotman et al., 2002). All mice used in this study were analyzed at 12 months of age and were littermates produced by crossing heterozygous Cln3<sup>ex7/8</sup> knock-in mice that were from an F4 CD1 backcross generation. Mice of both sexes were used for this analysis since we have previously observed no significant difference in JNCL-like phenotype between male and female Cln3<sup>ex7/8</sup> mice (Cotman et al., 2002). All perfusion procedures were carried out in accordance with NIH guidelines and under the animal care committee regulations at Massachusetts General Hospital.

Histological processing

For histological analysis, 12-month-old homozygous Cln3<sup>ex7/8</sup> knock-in mice and age-matched control littermates (n = 5) were fixed by transcardial perfusion. Mice were first deeply anaesthetized with sodium pentobarbitone (100 mg/kg) and transcardially perfused with vascular rinse (0.8% NaCl in 100 mM NaHPO<sub>4</sub>) followed by a freshly made and filtered solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were subsequently removed and weighed before overnight post-fixation and cryoprotection at 4°C in a solution containing 30% sucrose in Tris-buffered saline (TBS: 50 mM Tris, pH 7.6) with 0.05% NaN<sub>3</sub>. Brains were subsequently processed and sectioned as described previously (Bible et al., 2004; Pontikis et al., 2004), with serial 40 μm frozen coronal sections stored at −40°C in cryoprotectant solution (TBS/30% ethylene glycol/15% sucrose/0.05% sodium azide) prior to histological processing.

Nissl staining

To provide direct visualization of neuronal morphology every sixth section through the CNS was stained with the Nissl dye cresyl violet as previously described (Bible et al., 2004; Pontikis et al., 2004). Briefly, sections were mounted onto gelatin-chrome alum-coated Superfrost microscope slides (VWR, Dorset, UK), air dried overnight and incubated for 20 min at 55°C in 0.05% cresyl fast violet and 0.05% acetic acid (VWR, Dorset, UK), rinsed in distilled water and differentiated through a graded series of alcohol before clearing in xylene (VWR, Dorset, UK) and coverslipping with DPX (VWR, Dorset, UK). These Nissl-stained sections were subsequently used for stereological analysis of regional volume, cortical thinning, cortical and thalamic neuron number, as described previously (Bible et al., 2004; Pontikis et al., 2004).

To visualize autofluorescent storage material, selected sections from each animal were mounted upon Superfrost microscope slides (VWR, Dorset, UK) and immediately coverslipped with Vectashield aqueous mounting medium (Vector Laboratories, Peterborough, UK) (Bible et al., 2004; Griffey et al., 2003). Sections were viewed under conventional epifluorescence illumination on a Zeiss Axioskop2 MOT microscope (Carl Zeiss Ltd., Welwyn Garden City, UK) and images recorded at multiple wavelengths using a Zeiss AxioCam and Axiovision software (Carl Zeiss Ltd., Welwyn Garden City, UK).

Immunohistochemistry for interneuron markers

To survey representative interneuron populations in homozygous Cln3<sup>ex7/8</sup> mice, adjacent one-in-six series of sections were immunohistochemically stained for the neuropeptide somatostatin (SOM) or the calcium binding protein parvalbumin (PV), as previously described (Bible et al., 2004; Pontikis et al., 2004). Sections were incubated for 15 min in 1% H<sub>2</sub>O<sub>2</sub> in TBS, rinsed in TBS and blocked for 40 min in TBS/0.3% Triton X-100/15% normal goat serum (NGS) before overnight incubation at 4°C in the following polyclonal primary antisera (rabbit anti-SOM, Peninsula, CA, USA, 1:2000; rabbit anti-PV, Swant, Bellinzona, Switzerland, 1:5000) diluted in TBS with 10% normal goat serum and 0.3% Triton X-100. Sections were then rinsed in TBS and incubated for 2 h with secondary antisera (biotinylated goat anti-rabbit IgG, Vector Laboratories, UK, 1:1000) in TBS/0.3% Triton X-100/10% NGS. Following rinsing in TBS, sections were incubated for 2 h in an avidin–biotin–peroxidase complex in TBS ( Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Sections were next rinsed in TBS and immunoreactivity was visualized by incubation in 0.05% DAB (Sigma, Dorset, UK) and 0.001% H<sub>2</sub>O<sub>2</sub> in TBS for 10 min (a time which represents saturation for this reaction). Sections were then transferred to excess ice-cold TBS and were rinsed again, mounted, air dried, cleared in xylene and coverslipped with DPX (VWR, Dorset, UK).
Immunohistochemistry for glial markers

To assess the extent of glial activation in 12-month-old homozygous Cln3<sup>AxC<sub>7/8</sub></sup> knock-in mice, adjacent one-in-six series of free-floating frozen sections were immunohistochemically stained using the standard immunoperoxidase protocol described above for detection of astrocytic (GFAP, S100) and microglial (F4/80) markers using the following polyclonal primary antisera (polyclonal rabbit anti-cow GFAP, DAKO, Cambridge, UK, 1:1000; polyclonal goat anti-rabbit S100), DAKO, Cambridge, UK, 1:1000; monoclonal rat anti-mouse F4/80, Serotec, Oxford, UK, 1:100). Sections were then rinsed in TBS with subsequent incubation in secondary anti-serum (swine anti-rabbit [GFAP and S100], Vector Laboratories, Peterborough, UK, 1:400; and mouse adsorbed rabbit anti-rat [F4/80], Vector Laboratories, Peterborough, UK) followed by avidin–biotin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Immunoreactivity was visualized by a standard DAB reaction and sections were mounted onto slides, air dried, cleared in xylene and coverslipped with DPX (VWR, Peterborough, UK). To examine neuronal number and volume within individual cortical laminae, we used StereoInvestigator software to obtain unbiased optical fractionator estimates of neuronal number and nucleator estimates of neuronal volume from Nissl-stained sections. These estimates were obtained for neurons in lamina V of S1BF and M1 and of neurons in laminae II, III and IV in S1BF, together with afferent thalamic neurons in the ventral posterior nucleus (VPM/VPL). These measurements were performed as described previously (Bible et al., 2004), with a random starting section chosen, followed by every sixth Nissl-stained section thereafter. The boundaries of M1 and S1BF regions and each individual lamina were defined by reference to landmarks in Paxinos and Franklin (2001). Only neurons with a clearly identifiable nucleus were sampled and all counts were carried out using a ×100 oil objective (NA 1.4). The following sampling scheme was applied to the regions of interest. Grid area for lamina V M1 and S1BF 122,500 μm<sup>2</sup>, frame area 2141.3 μm<sup>2</sup>; grid area for S1BF laminae II and III of 122,500 μm<sup>2</sup>, frame area 2141.3 μm<sup>2</sup>; grid area for S1BF lamina IV of 40,000 μm<sup>2</sup>, frame area 1095.82 μm<sup>2</sup>; grid area for VPM/VPL of 122,500 μm<sup>2</sup>, frame area 3382.55 μm<sup>2</sup>. Measurements of total neuronal number and volume

To examine neuronal number and volume within individual cortical laminae, we used StereoInvestigator software to obtain unbiased optical fractionator estimates of neuronal number and nucleator estimates of neuronal volume from Nissl-stained sections. These estimates were obtained for neurons in lamina V of S1BF and M1 and of neurons in laminae II, III and IV in S1BF, together with afferent thalamic neurons in the ventral posterior nucleus (VPM/VPL). These measurements were performed as described previously (Bible et al., 2004), with a random starting section chosen, followed by every sixth Nissl-stained section thereafter. The boundaries of M1 and S1BF regions and each individual lamina were defined by reference to landmarks in Paxinos and Franklin (2001). Only neurons with a clearly identifiable nucleus were sampled and all counts were carried out using a ×100 oil objective (NA 1.4). The following sampling scheme was applied to the regions of interest. Grid area for lamina V M1 and S1BF 122,500 μm<sup>2</sup>, frame area 2141.3 μm<sup>2</sup>; grid area for S1BF laminae II and III of 122,500 μm<sup>2</sup>, frame area 2141.3 μm<sup>2</sup>; grid area for S1BF lamina IV of 40,000 μm<sup>2</sup>, frame area 1095.82 μm<sup>2</sup>; grid area for VPM/VPL of 122,500 μm<sup>2</sup>, frame area 3382.55 μm<sup>2</sup>. Measurements of total neuronal number and volume

The number of GABAergic interneurons expressing SOM and PV in M1 and S1BF was determined using the design-based optical fractionator method (West et al., 1991). Immunoreactive neurons were sampled using a series of counting frames distributed over a grid and superimposed onto the section using StereoInvestigator software. The rostral and caudal limits of M1 and S1BF regions were defined according to Paxinos and Franklin (2001). The lateral to medial extent of M1 and S1BF were identified by comparison with an adjacent series of Nissl-stained sections and anatomical reference points. A ×40 oil objective (NA 1.30) was then used to count clearly identifiable immunoreactive neurons, which fell within the dissector frame. The following sampling scheme was applied to the regions of interest; Grid area for SOM and PV in M1 and S1BF 38,275 μm<sup>2</sup>, frame area 20,191 μm<sup>2</sup>. Measurements of interneuron number

The number of GABAergic interneurons expressing SOM and PV in M1 and S1BF was determined using the design-based optical fractionator method (West et al., 1991). Immunoreactive neurons were sampled using a series of counting frames distributed over a grid and superimposed onto the section using StereoInvestigator software. The rostral and caudal limits of M1 and S1BF regions were defined according to Paxinos and Franklin (2001). The lateral to medial extent of M1 and S1BF were identified by comparison with an adjacent series of Nissl-stained sections and anatomical reference points. A ×40 oil objective (NA 1.30) was then used to count clearly identifiable immunoreactive neurons, which fell within the dissector frame. The following sampling scheme was applied to the regions of interest; Grid area for SOM and PV in M1 and S1BF 38,275 μm<sup>2</sup>, frame area 20,191 μm<sup>2</sup>. Due to the comparatively low abundance of interneurons present in the hippocampus versus the neocortex, stereological methods prove inefficient at estimating hippocampal interneuron numbers without sampling the entire tissue (Bible et al., 2004). Instead, counts of the number of interneurons expressing SOM or PV were made, as described previously (Bible et al., 2004; Cooper et al., 1999; Pontikis et al., 2004) in defined hippocampal subfields. Counts were carried out under a ×20 objective and only positively stained cells with clear neuronal morphology were counted. The number of interneurons in each hippocampal subfield was expressed as the mean number of neurons per section. Quantitative analysis of glial phenotype

The optical density of GFAP and F4/80 immunoreactivity was assessed using a semi-automated thresholding image analysis system (Optimas, Media Cybernetics, Silver Springs, MD). This analysis was performed blind to genotype, as previously described (Bible et al., 2004; Pontikis et al., 2004). Forty non-overlapping images were captured, on three consecutive sections, through the cortical regions M1, S1BF and V1, stratum, hippocampal dentate gyrus and a combined measurement for the stratum oriens and
CA1. All RGB images were captured via a live video camera (JVC, 3CCD, KY-F55B), mounted onto a Zeiss Axioplan microscope using a ×40 objective and saved as JPEGs. All parameters including lamp intensity, video camera setup and calibration were maintained constant throughout image capturing.

Images were subsequently analyzed using OPTIMAS 6.2 image analysis software (Media Cybernetics, Silver Springs, MD), using an appropriate threshold that selected the foreground immunoreactivity above background. This threshold was then applied as a constant to all subsequent images analyzed per batch of animals and reagent used to determine the specific area of immunoreactivity for each antigen in each region. Each field measured 120 μm wide, with a height of 90 μm. Therefore, the total area compiled from 40 fields in each region corresponded to 432,000 μm². Macros were recorded to transfer the data to a Microsoft Excel spreadsheet and were subsequently analyzed statistically. Data were plotted graphically as the mean percentage area of immunoreactivity per field ± SEM for each region.

Measurements of astrocytic number

To quantify changes in astrocyte number we obtained design-based optical fractionator estimates of S100β-positive soma, together with nucleator estimates of astrocyte volume. Immunoreactive astrocytes were sampled in S1BF and CA1/stratum oriens using a series of counting frames distributed over a grid and superimposed onto the section using StereoInvestigator software, exactly as described previously (Bible et al., 2004). The rostral and caudal limits of S1BF and CA1/stratum oriens regions were defined as described above. A ×40 oil objective (NA 1.30) was then used to count clearly identifiable immunoreactive astrocytes, which fell within the dissector frame. The following sampling scheme was applied to the regions of interest; Grid area for S100β in S1BF and CA1/oriens 20,191 μm², frame area 122,500 μm².

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 coefficient of error (CE) for all individual optical fractionator and nucleator estimates was calculated according to the method of Gundersen and Jensen (1987) and was less than 0.08 in all these analyses.

Results

Homozygous Cln3Δex7/8 mice exhibit widespread accumulation of autofluorescent storage material

Macroscopic examination of the brains of 12-month-old Cln3Δex7/8 homozygotes did not reveal an obvious degenerative phenotype, with the cortex and cerebellum of affected mice appearing indistinguishable from controls (data not shown). Consistent with these observations, the brains of homozygous Cln3Δex7/8 mice were lighter than age-matched controls, but this reduction did not reach statistical significance (control 494 ± 43 mg; Cln3Δex7/8 456 ± 39 mg, P = 0.128, n = 5). Microscopically, homozygous Cln3Δex7/8 mice exhibited widespread intracellular accumulation of autofluorescent storage material within neuronal soma throughout the CNS (Fig. 1). In mutant mice, this storage material was present as punctate material that fluoresced at multiple wavelengths, as viewed by conventional epifluorescence microscopy (Figs. 1B–D, F–G). In contrast, sections from littermate controls display a low level of background tissue fluorescence and many fewer scattered deposits of storage material within the cortex (A), and a low level of autofluorescence within Purkinje neurons of the cerebellum (E).
contrast, control littermates displayed a low level of background autofluorescence and an age-related and sparsely scattered accumulation of autofluorescent material (Fig. 1A) that was more pronounced in Purkinje neurons of the cerebellum (Fig. 1E).

**Lack of significant regional atrophy in homozygous Cln3<sup>dex7/8</sup> knock-in mice**

Since homozygous Cln3<sup>dex7/8</sup> brains did not display an overt phenotype macroscopically, we carried out a stereological survey of regional volume and cortical thickness to look for more subtle neurodegenerative changes in Nissl-stained sections. Cavalieri estimates of regional volume revealed that although many CNS regions including the cortex, hippocampus and cerebellum were reduced in size in Cln3<sup>dex7/8</sup> mice, the thalamus was the only structure that displayed significantly reduced volume in homozygous Cln3<sup>dex7/8</sup> mice (Fig. 2).

**Regional effects upon cortical thinning and lamination in homozygous Cln3<sup>dex7/8</sup> mice**

Because measurements of overall cortical volume cannot discriminate between events in different cortical subfields, we carried out a series of cortical thickness measurements of primary motor (M1), somatosensory barrel field (S1BF), primary visual (V1) and lateral entorhinal (Lent) regions (Fig. 3). Homozygous Cln3<sup>dex7/8</sup> mice showed variable effects upon cortical thickness in different regions. There was significant thinning of S1BF and Lent cortex, but a significant increase in the thickness of M1 (Fig. 3A). In contrast, V1 exhibited less pronounced thinning in homozygous Cln3<sup>dex7/8</sup> mice that was not significant versus littermate controls (Fig. 3A).

To determine whether these changes were due to laminar-specific events, we made measurements of individual lamina thickness (Figs. 3B–E). Although significant reductions in the thickness of lamina I were consistently seen in S1BF, V1 and Lent and the thickness of laminae II + III in M1, S1BF and Lent (Figs. 3B–D), the remaining laminae in these cortical regions displayed a complex series of contrasting changes in thickness (Table 1). The most extreme example was in Lent which displayed significant reductions in the thickness of laminae I and II, but significantly increased thickness of laminae III and IV (Fig. 3D). The increase in the thickness of M1 in homozygous Cln3<sup>dex7/8</sup> mice appeared to be largely due to a significant increase in the thickness of lamina V (Fig. 3E), whereas all other laminae were either unchanged (I and VI) or displayed a small, but significant thinning (II + III).

**Regional and laminar effects upon neuronal number in homozygous Cln3<sup>dex7/8</sup> mice**

To investigate whether these changes in individual laminar thickness in homozygous Cln3<sup>dex7/8</sup> mice were the result of effects upon alterations in neuronal number and/or neuronal size, we obtained optical fractionator estimates of neuronal number and nucleator estimates of neuronal volume in Nissl-stained sections. These data were collected for lamina V pyramidal neurons in S1BF and M1, two cortical regions which displayed contrasting changes in the thickness of this lamina, in addition to lamina IV granule neurons of S1BF and a combined measure of laminae II and III neurons in S1BF (Fig. 4).

Although there was a trend to reduced neuronal number, lamina V pyramidal neurons were not significantly lost in either M1 or S1BF of homozygous Cln3<sup>dex7/8</sup> mice (Fig. 4A). In contrast, there was a significant loss of neurons in laminae II and III of S1BF of mutant mice, together with a significant loss of granule neurons in lamina IV of this cortical region (Fig. 4A). Nucleator estimates of neuronal volume revealed only minor changes in cell size in individual laminae (Fig. 4B), none of which reached statistical significance.

To determine the effect of this loss of granule neurons in S1BF upon afferent thalamic neurons, we obtained unbiased stereological estimates of neuronal number and volume in the ventral posterior thalamic nucleus (VPM/VPL). These analyses revealed a significant loss of neurons within VPM/VPL of homozygous Cln3<sup>dex7/8</sup> mice (Fig. 4A), although these neurons were not significantly hypertrophied (Fig. 4B).
Survival of GABAergic interneurons in homozygous Cln3<sup>Δex7/8</sup> mice

Loss of GABAergic interneurons is a common feature of other murine models of NCL (Cooper, 2003; Mitchison et al., 2004). To determine whether these neuronal populations were also affected in homozygous Cln3<sup>Δex7/8</sup> mice, the expression of parvalbumin (PV) and somatostatin (SOM) were surveyed in the cortex (Fig. 5A) and hippocampus (Figs. 5B and C). These markers are usually expressed in representative interneuron populations (Freund and Buzsáki, 1996), which are consistently affected in mouse models of infantile NCL (Bible et al., 2004; Jalanko et al., 2005), juvenile NCL (Mitchison et al., 1999; Pontikis et al., 2004) and variant late infantile NCL (Cooper et al., 1999; Kopra et al., 2004).

Primary motor and somatosensory cortex

Optical fractionator estimates revealed a general trend to a reduced number of interneurons immunoreactive for PV and SOM in M1 and S1BF, but this loss of interneurons did not reach significance for interneurons stained for either antigen in these cortical regions (Fig. 5A).

Hippocampus

We next examined interneuron populations that are immunoreactive for these markers in the hippocampal formation of homozygous Cln3<sup>Δex7/8</sup> mice. There was an overall trend to reduced number of SOM-positive interneurons in homozygous Cln3<sup>Δex7/8</sup> mice, although this did not reach significance in any hippocampal subfield (Fig. 5C). Effects upon the number of PV-
positive interneurons were more variable between hippocampal subfields in homozygous Cln3<sup>Δex7/8</sup> mice, but none of these changes reached statistical significance (Fig. 5B).

**Table 1**

Tabular depiction of the significant changes in individual laminar thickness in the primary motor (M1), somatosensory barrel field (S1BF), primary visual (V1) and entorhinal cortex (Lent) of 12-month-old Cln3<sup>Δex7/8</sup> compared with age-matched controls (+/+).

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Significantly ↑ = thicker in Cln3<sup>Δex7/8</sup>; ↓ = significantly thinner in Cln3<sup>Δex7/8</sup>; NS = not significant; na = not applicable, M1 has no lamina IV. A combined measurement of laminae II and III was made in all regions except Lent where these laminae were measured separately.

**Fig. 4.** Thalamocortical neuron loss in homozygous Cln3<sup>Δex7/8</sup> mice. (A) Optical fractionator estimates of neuronal number revealed the significant loss of neurons in laminae II and III and lamina IV granule neurons, but no significant loss of lamina V pyramidal neurons in primary somatosensory barrel field cortex (S1BF) or primary motor cortex (M1) of homozygous Cln3<sup>Δex7/8</sup> mice compared with littermate controls (+/+). There was also a significant reduction in the number of neurons in the ventral posterior thalamic nucleus (VPM/VPL) of mutant mice. (B) Nucleator estimates of neuronal volume revealed no significant atrophy or hypertrophy in any laminae of Cln3<sup>Δex7/8</sup> homozygotes. *P < 0.05, **P < 0.001, one-way ANOVA.

**Fig. 5.** Interneuron populations are not significantly affected in homozygous Cln3<sup>Δex7/8</sup> mice. (A) Persistence of cortical interneurons in aged Cln3<sup>Δex7/8</sup> homozygotes. Optical fractionator estimates of parvalbumin (PV) and somatostatin (SOM)-positive interneuron number in the primary motor (M1) and somatosensory barrel field (S1BF) cortex reveal no significant loss of these neurons in 12-month-old Cln3<sup>Δex7/8</sup> homozygotes compared with littermate controls (+/+). (B–C) Survival of hippocampal interneurons in aged homozygous Cln3<sup>Δex7/8</sup> mice. Counts of hippocampal interneuron number revealed no significant loss of interneurons immunoreactive for either parvalbumin (B) or somatostatin (C) in any subfield of 12-month-old Cln3<sup>Δex7/8</sup> and age-matched controls (+/+).

Regionally restricted astrocytic and microglial activation in homozygous Cln3<sup>Δex7/8</sup> mice

To examine glial responses in homozygous Cln3<sup>Δex7/8</sup> mice, we first surveyed the distribution of cells immunoreactive for the astrocytic markers GFAP and S100β, in addition to the microglial marker F4/80. Subsequently, we used image analysis to quantify the expression of these markers in the striatum, cortical subregions...
M1 and S1BF; and hippocampal subfields hilus, CA1, CA2 and CA3 using standard methods (Bible et al., 2004; Pontikis et al., 2004). Reactive astrogliosis may represent hypertrophy of astrocytes (thickening of processes and changes in cell body volume) which may be independent of cell proliferation. Therefore, we also obtained optical fractionator estimates of the number of astrocytes immunoreactive for S100\(_\beta\), a calcium binding protein that is expressed predominantly in astrocytes (Boyes et al., 1986).

GFAP- and S100\(_\beta\)-positive astrocytes

In the neocortical grey matter of control mice, only few GFAP-positive astrocytes were present, predominantly in lamina VI and in lamina I adjacent to the pial surface (Fig. 6A). In marked contrast, homozygous Cln3\(^{\Delta ex7/8}\) mice displayed a profound astrogliosis across the cortical mantle with intensely GFAP-immunoreactive astrocytes present in both superficial and deeper cortical laminae (Fig. 6B), with positive astrocytes extending in clusters across all laminae. At higher power, these GFAP-positive astrocytes displayed marked hypertrophy with numerous thickened and branched processes and enlarged soma in homozygous Cln3\(^{\Delta ex7/8}\) mice compared to control littermates (Figs. 6A and B). In marked contrast to GFAP, S100\(_\beta\) immunoreactivity within the neocortex was present within numerous intensely stained cell somas that were distributed evenly across laminae and did not differ obviously between animals of either genotype (Figs. 6C and D).

Subcortically, this astrocytic response was far less pronounced with reduced staining for GFAP present within the hippocampus and cerebellum (data not shown). In contrast, the thalamus of homozygous Cln3\(^{\Delta ex7/8}\) mice exhibited pronounced GFAP immunoreactivity compared to controls (Figs. 6E and F). However, this staining for GFAP was not uniformly distributed but was highest in individual thalamic nuclei, most notably within the ventral posterior (VPM/VPL), lateral regions of the laterodorsal (LD)
and the mediadorsal (MD) thalamic nuclei, but virtually absent in the adjacent ventromedial (VM), posterior (Po) or intralaminar thalamic nuclei (Fig. 6F).

**Quantitative analysis of astrocytosis**

Consistent with these morphologic observations, quantitative image analysis revealed a significant and widespread increase in GFAP expression in both superficial and deeper cortical layers (Fig. 7A). This upregulation was not confined to any cortical region with similar increases in GFAP staining in M1, S1BF, V1 and Lent (Fig. 7A). In contrast to these events in the neocortex, quantitative analysis revealed a range of different astrocyte responses in the hippocampus (Fig. 7B), cerebellum (Fig. 7C) and subcortical structures of Cln3<sup>-exo7/8</sup> homozygotes. Unexpectedly, the hippocampal dentate

![Figure 7](image.png)

**Fig. 7. Regionally variable astrocytosis in homozygous Cln3<sup>-exo7/8</sup> mice.** (A) Quantitative image analysis reveals the widespread and significantly increased expression of GFAP in the superficial and deep cortical laminae of Cln3<sup>-exo7/8</sup> homozygotes compared with littermate controls (+/+) at 12 months of age. (B–C) In contrast, hippocampal (B) and cerebellar grey matter (C) display significantly reduced levels of GFAP in mutant mice compared with controls. Areas surveyed include striatum (CPu), hippocampal dentate gyrus (DG), hippocampal stratum oriens and CA1 (Oriens CA1), cerebellar white matter (WM) and molecular and granule cell layers (Mol + Gr) in the lateral hemispheres (Lat) and vermis (Verm). (D) Optical fractionator estimates revealed no significant change in the number of S100β-positive astrocytes in either the primary somatosensory barrel field cortex (S1BF) or hippocampal CA1 and stratum oriens of homozygous Cln3<sup>-exo7/8</sup> mice, compared with littermate controls (+/+). ***P < 0.001, one-way ANOVA.
gyrus, stratum oriens and CA1 of homozygous Cln3 \textsuperscript{Δex7/8} mice exhibited significantly reduced GFAP expression compared to control littermates (Fig. 7B). GFAP expression in homozygous Cln3 \textsuperscript{Δex7/8} mice was similarly reduced in the grey matter of cerebellar lateral hemisphere and vermis, but was unchanged in the white matter within these regions of the cerebellum (Fig. 7C).

To determine whether these changes in GFAP expression reflected changes in the number of astrocytes, we next obtained unbiased stereological estimates of the number of S100\textsubscript{β}-positive astrocytes. These optical fractionator data were collected within one representative region that displayed increased GFAP expression (S1BF) and one that displayed decreased GFAP expression (stratum oriens and CA1 of the hippocampus) but did not reveal any significant change in astrocyte number within either region of homozygous Cln3 \textsuperscript{Δex7/8} mice compared with control littermates (Fig. 7D).

**F4/80-positive microglia**

Compared to control littermates, F4/80 immunoreactive microglia were consistently more prominent in homozygous Cln3 \textsuperscript{Δex7/8} mice, with these differences again more prominent in the neocortex than other CNS regions (Fig. 8). In the neocortex of control littermates, the widespread distribution of microglia was revealed by pale F4/80 immunoreactivity within cell soma and thin processes extending into the neuropil (Figs. 8A and C). In contrast, throughout the cortical mantle of Cln3 \textsuperscript{Δex7/8} homozygotes, there were intensely F4/80 immunoreactive microglia with numerous ramified processes, often with more prominent cell soma (Figs. 8B and D). In homozygous Cln3 \textsuperscript{Δex7/8} mice, these F4/80 immunoreactive microglia were present with no particular focus and across all laminae, although F4/80-positive cells frequently displayed more morphological signs of activation in deeper laminae with fewer thickened processes.

Fig. 8. Activation of microglia in homozygous Cln3 \textsuperscript{Δex7/8} mice. Immunohistochemical staining for F4/80 revealed graded activation of microglia in 12-month-old homozygous Cln3 \textsuperscript{Δex7/8} mice compared with littermate controls (+/+). At this age, F4/80-positive microglia with numerous ramified processes in the primary motor cortex (M1) were more darkly stained in Cln3 \textsuperscript{Δex7/8} homozygotes (B, D) than littermate controls (A, C). This difference between genotypes was less pronounced in the striatum (CPu), although numerous partially activated microglia with enlarged soma and short thickened processes were evident in homozygous Cln3 \textsuperscript{Δex7/8} mice (F) compared to the microglia with thin ramified processes that were present in littermate controls (E).
Differences in the relative intensity of F4/80 staining between genotypes were less marked in subcortical structures (Figs. 8E and F), although homozygous Cln3<sup>ex7/8<sup> mice again displayed more morphological evidence of microglial activation in these regions. Although complete morphological transformation to brain macrophage-like morphology in homozygous Cln3<sup>ex7/8<sup> mice was seldom seen, these mice frequently displayed F4/80 immunoreactive microglia with enlarged soma and numerous short thickened processes (Fig. 8F), compared to the threadlike and ramified processes in control mice (Fig. 8E).

**Discussion**

This study represents the first detailed description of the CNS of homozygous Cln3<sup>ex7/8<sup> mice, a model that recapitulates the major deletion present in the vast majority of JNCL alleles (Cotman et al., 2002). Our data revealed no interneuron loss or significant regional atrophy in Cln3<sup>ex7/8<sup> homozygotes by 12 months of age. The marked exception was the thalamus, which displayed regional atrophy and a significant loss of somatosensory relay neurons in mutant mice, together with loss of their target neurons within the somatosensory cortex. These findings provide significant new data of neuronal loss within the thalamocortical system in JNCL, which was accompanied by a pronounced astrocytic response within individual thalamic nuclei of Cln3<sup>ex7/8<sup> homozygous mice. Astrocytic activation was prominent cortically, yet was diminished within the hippocampus and cerebellum of mutant mice. These data provide further evidence that both neuronal and glial responses in JNCL are localized and regionally specific, as typified by our novel findings within the thalamocortical system of homozygous Cln3<sup>ex7/8<sup> mice.

**Homozygous Cln3<sup>ex7/8<sup> mice as a model for JNCL**

Bearing the major deletion that is present in over 85% of CLN3 alleles in JNCL (International Batten Disease Consortium, 1995), Cln3<sup>ex7/8<sup> knock-in mice are an accurate model of this disorder (Cotman et al., 2002). Certainly, the homozygous Cln3<sup>ex7/8<sup> mice used in this study exhibit an early onset of neurologic deficits (Cotman et al., 2002) that are more pronounced than the relatively mild retinal and neurological phenotypes of Cln3<sup>ex7/8<sup> mice (Katz et al., 1999; Mitchison et al., 1999; Seigel et al., 2002). Nevertheless, any comparisons between these mice are complicated by the different strain backgrounds these models have been raised on (Mitchison et al., 1999; Cotman et al., 2002). As such, it will be essential to re-evaluate their phenotypes once these mouse models of JNCL are all available upon a common strain background.

**Selective loss of cortical neurons in homozygous Cln3<sup>ex7/8<sup> mice**

Although cortical atrophy is a consistent feature of various mouse models of NCL, including Cln3<sup>−/−<sup> (Mitchison et al., 1999; Pontikis et al., 2004), PPT1<sup>−/−<sup> (Bible et al., 2004) and TPP1-deficient mice (Sleat et al., 2004), atrophy of the neocortex of homozygous Cln3<sup>ex7/8<sup> mice was relatively mild and may only become more pronounced with increased age. Indeed, since approximately 20% of these mice do not reach 12 months of age (Cotman et al., 2002), it is possible that by performing our analysis at this age, we have selected mice that may present with a less pronounced or delayed neurodegenerative phenotype.

Our data from 12-month-old homozygous Cln3<sup>ex7/8<sup> knock-in mice did reveal a range of selective effects upon cortical thickness that varied between different subfields (Fig. 3). These effects extended to individual laminae and, as highlighted by data from the somatosensory cortex of homozygous Cln3<sup>ex7/8<sup> mice, to neuronal number within these laminae (Fig. 4). The most pronounced loss within this cortical region was of laminae II and III neurons, which supply commissural and association projections to other areas of cortex. The loss of these neurons may potentially have significant consequences for coordinating neuronal activity between hemispheres and cortical regions. In this respect, it will be important to determine the functional correlates of these pathological changes in the cortex of homozygous Cln3<sup>ex7/8<sup> mice.

Although GABAergic interneuron subpopulations were depleted to different extents in various cortical and hippocampal subfields, unlike human JNCL (Tynelä et al., 2004), the Cln3<sup>ex7/8<sup> model of JNCL (Mitchison et al., 1999; Pontikis et al., 2004) or other mouse models of NCL (Cooper et al., 1999; Bible et al., 2004; Kopra et al., 2004; Jalanko et al., 2005), these cell populations were not significantly affected in homozygous Cln3<sup>ex7/8<sup> mice. The lack of significant loss of these interneurons in Cln3<sup>ex7/8<sup> homozygotes may be characteristic of these mice, or alternatively, this phenotype may take longer to develop.

**Loss of thalamic neurons in homozygous Cln3<sup>ex7/8<sup> mice**

Our analysis of regional volume was informative revealing thalamic atrophy in homozygous Cln3<sup>ex7/8<sup> mice, a phenotype that is also apparent in PPT1-deficient mice (Bible et al., 2004). Although the thalamus typically displays a reduced MRI signal intensity in symptomatic INCL and JNCL patients (Autti et al., 1997; Vanhanen et al., 2004), the cellular basis of this hypointensity has remained unknown. Indeed, in contrast to the wealth of information available for the cortex and hippocampus (Braak and Goebel, 1978, 1979; Haltia, 2003; Haltia et al., 2001; Tynelä et al., 2004), neuropathological data of neuronal loss within subcortical structures in JNCL are extremely limited (Braak and Braak, 1987; Braak et al., 1979). As such, our data from homozygous Cln3<sup>ex7/8<sup> mice are significant in providing the first direct evidence for the loss of thalamic relay neurons in this disorder. It is unclear whether thalamic neurons are themselves inherently vulnerable in JNCL, or degenerate in response to events within the cortex. Determining the precise sequence of events in the thalamocortical system during pathogenesis will be crucial to distinguish between these distinct mechanistic possibilities.

The clinical consequences of these events within the thalamocortical system remains unclear, although progressive changes in somatosensory evoked potentials are consistently reported in multiple forms of NCL (Tackmann and Kuhlendahl, 1979; Verrecuyssen et al., 1982; Vanhanen et al., 1997; Lautonen et al., 1997), including JNCL (Lautonen et al., 1997, 1999). Indeed, it has been suggested that progressive thalamic dysfunction may also contribute to the sleep disturbances that are characteristic of the NCLs (Vanhanen et al., 1997). It will be highly informative to determine whether the loss of thalamic neurons and/or their cortical targets contributes to similar phenotypes in homozygous Cln3<sup>ex7/8<sup> knock-in mice.

We have already reported motor coordination problems by 12 months of age in homozygous Cln3<sup>ex7/8<sup> knock-in mice (Cotman et al., 2002), but this current study demonstrated no overt effects within the cerebellum of these mice. Although subtle effects on
other neuronal populations may also be present, our data suggest that neurologic problems in homozygous Cln3<sup>ex7/8</sup> knock-in mice may involve altered sensory feedback through the thalamus. In this context, it will be important to correlate sensory behaviors and other forms of neurological impairment with pathological changes in the thalamocortical system during disease progression.

**Prominent astrocytosis and graded microglial responses in Cln3<sup>ex7/8</sup> homozygotes**

Prominent gliosis is a consistent feature of autopsy material from individuals with JNCL (Braak and Goebel, 1978, 1979; Tyynelä et al., 2004) and other forms of NCL (Haltia et al., 1973a,b; Herva et al., 2000; Tyynelä et al., 1997, 2004). Similar reactive events are evident in mouse models of CLN8 (Cooper et al., 1999), and infantile NCL (Bible et al., 2004; Jalanko et al., 2005), but a considerably more subtle glial phenotype is evident in Cln3<sup>−/−</sup> (Pontikis et al., 2004). Markers of astrocytic and microglial activation are significantly elevated by 5 months of age in Cln3<sup>−/−</sup>, but this activation remains at a low level with no morphological transformation to brain macrophages or obvious astrocytic hypertrophy (Pontikis et al., 2004).

Our data from Cln3<sup>ex7/8</sup> homozygotes provide a distinctly different picture of reactive changes in these mice, with widespread astrocyte hypertrophy across the cortical mantle and variable responses subcortically (Fig. 6). Curiously, there was little evidence of astrocytic proliferation or hyperplasia in homozygous Cln3<sup>ex7/8</sup>/C0 mice, with no difference in the number of S100β-positive astrocytes between animals of either genotype. These S100β data also suggest that reduced GFAP immunoreactivity evident in the cerebellum and hippocampus of homozygous Cln3<sup>ex7/8</sup>/C0 mice appears to represent downregulation of this marker rather than the targeting of astrocytes that has been proposed in Cln3<sup>−/−</sup> (Pontikis et al., 2004).

Although astrocytes classically mediate responses to neuronal injury (Raivich et al., 1999), emerging evidence suggests these cell types play dynamic roles in regulating fluid homeostasis (Simard and Nedergaard, 2004) and controlling local concentrations of glutamate, influencing neuronal activity and synaptic efficacy (Oliet et al., 2001; Piet et al., 2002). Although the localized events that trigger astrocytosis in homozygous Cln3<sup>ex7/8</sup>/C0 mice remain unclear, these may involve neuronal damage due to accumulated storage material. However, in contrast to the widespread accumulation of storage material (Fig. 1), astrocytic activation within the thalamus was restricted to individual nuclei (Fig. 6), with particularly prominent GFAP staining within the VPM/VPL which also exhibited neuronal loss (Fig. 4). These reactive changes may represent a localized protective response to the effects of the Cln3 mutation, as has been suggested in human JNCL (Tyynelä et al., 2004). In this respect, investigating the precise spatiotemporal relationship between astrocytosis and neuronal loss at different stages of disease progression is likely to be highly informative.

In contrast to these prominent astrocytic responses in Cln3<sup>ex7/8</sup>/C0 homozygotes, the microglial responses in these mice were markedly less pronounced. The activation of microglia is a progressive and graded phenomenon that takes place in different ‘stages’ from extensively ramified cells to full blown brain macrophages (Raivich et al., 1999), and has been considered a sensitive marker of local neuronal damage (Streit, 2000, 2002). In Cln3<sup>ex7/8</sup> homozygous mice this microglial activation rarely reached its full extent to reveal brain macrophage morphology, even in areas where a profound astrocytic response was evident. These data suggest that whatever underlying molecular cues trigger astrocytosis in Cln3<sup>ex7/8</sup>/C0 knock-in mice, these are not sufficient to promote full morphological transformation of microglia. Alternatively, microglia may themselves be targeted by the effects of Cln3 mutation as has been suggested in Cln3<sup>−/−</sup> mice (Pontikis et al., 2004).

Taken together with data from other mouse models of NCL (Cooper et al., 1999; Lam et al., 1999; Mitchison et al., 1999; Bible et al., 2004), sheep with NCL (Oswald et al., 2001, in press) and human NCL (Tyynelä et al., 2004), our data suggest that highly regionalized interactions occur between neurons and glia at different stages of disease progression. Whether these events contribute directly to subsequent neurodegeneration has not been demonstrated, but the suggestion that neuron–glial interactions differ markedly between CNS regions may provide important clues to the pathogenesis of these disorders.

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**References**


