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

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ABSTRACT

Finnish variant LINCL (vLINCL$_{Fin}$) 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 vLINCL$_{Fin}$, 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 (Siintola et al., 2006, 2007).

The Finnish variant form of late infantile neuronal ceroid lipofuscinosis (vLINCLFin, 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). vLINCL$_{Fin}$ 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 vLINCL$_{Fin}$ 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

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thalamocortical system displays a series of localized neurodegenerative and reactive changes in other models of NCL (Pontikis et al., 2005; Weimer et al., 2006; Kielar 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 strain 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*$^{\Delta ex4}$) 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 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; Kielar 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 from 40 μm Nissl-stained cryosections, as described previously (Bible et al., 2004; Pontikis et al., 2004). A sampling grid with appropriate spacing was superimposed over each section, and the number of points covering the relevant areas counted using objectives of appropriate magnification and *StereoInvestigator* software (Microbrightfield Inc., Williston, VT). Regional volumes were expressed in μm^3 and the mean volume of each region was calculated for the *Cln5*$^{-/-}$ mice and controls.

Cortical thickness measurements

To explore the extent of cortical atrophy, cortical thickness measurements were made on the same one-in-six series of Nissl stained sections for primary motor (M1), primary somatosensory (S1BF), primary visual (V1) and lateral entorhinal (LEnt) cortex using an ×1.25 objective as described previously (Bible et al., 2004; Pontikis et al., 2004), using anatomical landmarks defined in Paxinos and Franklin (2001). On each of three consecutive sections, the length of perpendicular lines extending from the white matter to the pial surface was measured by placing ten evenly spaced lines spanning each cortical region. Results were expressed as mean cortical thickness in μm per region for *Cln5*$^{-/-}$ mice and controls. Individual laminar thicknesses were also measured in M1, S1BF, V1 and LEnt 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; Kielar 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 asses 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 Vectastain 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