A knock-in reporter mouse model for Batten disease reveals predominant expression of *Cln3* in visual, limbic and subcortical motor structures

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ABSTRACT

Juvenile neuronal ceroid lipofuscinosis (JNCL) or Batten disease is an autosomal recessive neurodegenerative disorder of children caused by mutation in *CLN3*. JNCL is characterized by progressive visual impairment, cognitive and motor deficits, seizures and premature death. Information about the localization of CLN3 expressing neurons in the nervous system is limited, especially during development. The present study has systematically mapped the spatial and temporal localization of CLN3 reporter neurons in the entire nervous system including retina, using a knock-in reporter mouse model. CLN3 reporter is expressed predominantly in post-migratory neurons in visual and limbic cortices, anterior and intralaminar thalamic nuclei, amygdala, cerebellum, red nucleus, reticular formation, vestibular nuclei and retina. CLN3 reporter in the nervous system is mainly expressed during the first postnatal month except in the dentate gyrus, parasolitary nucleus and retina, where it is still strongly expressed in adulthood. The predominant distribution of CLN3 reporter neurons in visual, limbic and subcortical motor structures correlates well with the clinical symptoms of JNCL. These findings have also revealed potential target brain regions and time periods for future investigations of the disease mechanisms and therapeutic intervention.

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Introduction

Neuronal ceroid lipofuscinoses (NCL) are a group of recessively inherited neurodegenerative diseases affecting the nervous system. The juvenile form of NCL (JNCL) is the most common form of NCL in children. The initial symptom of JNCL is progressive visual deterioration beginning at age 5–10 years, followed by mental retardation, seizure, psychomotor decline and finally premature death (Boustany, 1992; Goebel and Wisniewski, 2004). A pathological feature of NCL is autofluorescent storage material in neurons and many other cell types, although few non-neuronal symptoms have been reported (Hofman et al., 2001). The autofluorescent deposits in JNCL often appear as fingerprint-like by electron microscopy and contain mitochondrial ATP synthase subunit c as a major protein component (Mitchison et al., 2004; Mole et al., 2005; Phillips et al., 2005).

JNCL is caused by mutation in *CLN3* (Batten Disease Consortium, 1995), which encodes a 438-amino acid predicted transmembrane protein (CLN3, CLN3p or battenin). The function of CLN3 is not yet clear, nor is the pathogenesis of JNCL. Findings in the budding yeast suggest a role for Btn1p, the CLN3 ortholog, in lysosomal pH homeostasis (Pearce, 2000; Phillips et al., 2005), and arginine transport (Kim et al., 2003). Studies in the fission yeast similarly indicate a role for Btn1p in regulating vacuolar pH (Gachet et al., 2005), and additionally indicate a need for Btn1p for normal cell wall deposition and polarized cell growth (Codlin et al., 2008a; Codlin et al., 2008b), vacuolar protein sorting (Codlin and Mole, 2009), and more recently glucose and amino acid metabolism (Pears et al., 2010). In mammalian cells, CLN3 has been suggested to have anti-apoptotic properties (Narayan et al., 2006; Puranam et al., 1999), to participate in autophagic vacuolar maturation (Cao et al., 2006), endocytosis or vesicular trafficking (Luiro et al., 2004; Fossale et al., 2004; Metcalf et al., 2008) and our unpublished observations), or lipid metabolism or transport (Hobert and Dawson, 2007; Rusyn et al., 2008).

Perhaps reflective of its seemingly multifunctional nature, various subcellular localizations have been reported for CLN3 in cultured cells. In mammalian cells, CLN3 has been localized to lysosomes (Kyttala et al., 2004) or endosomes, plasma membrane and Golgi (Persaud-Sawin et al., 2004). Early works in neuronal cells co-localized CLN3 with

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synaptic vesicles (Haskell et al., 2000; Järvelä et al., 1999), or to non-synaptic vesicles along neurites (Luiro et al., 2001). In yeast, Btn1p has been localized to the vacuole in *S. cerivisiae* (Padilla-Lopez and Pearce, 2006) and to the cis Golgi in *Schizosaccharomyces pombe* (Codlin and Mole, 2009).

Recently, we have established a knock-in reporter mouse model for JNCL (Eliason et al., 2007). In this model a transgene encoding nuclear-targeted bacterial ß-galactosidase (ß-gal) was knocked into the mouse *Cln3* locus using targeted recombination, such that ß-gal transcription is controlled by native sequence 5' to the *Cln3* coding region, and ß-gal replaces much of exon 1 and all of exons 2–8. CLN3 is not expressed from the recombinant allele and the homozygous *Cln3*[LacZ/LacZ] mice are CLN3 null and serve as a JNCL model. Other JNCL mouse models include another CLN3-null mouse (Mitchison et al., 1999), as well as models designed to resemble the common human exon 7/8 deletion (Katz et al., 1999; Cotman et al., 2002). The earliest pathological feature reported in JNCL mouse models is autofluorescent inclusions, appearing embryonically in some regions (Cotman et al., 2002). Autofluorescence is widespread and progressive, becoming increasingly abundant in many brain areas from 3 to 12 months of age in CLN3 null mice (Mitchison et al., 1999). In our *Cln3*[lacz/lacz] mice, inclusions are prominent in neurons of the hippocampus and thalamus (Eliason et al., 2007). Gross anatomy and architecture of the CNS appears normal in JNCL mice, but quantitative histological techniques have revealed mild to moderate reduction in neuron numbers within some regions in adult mice. In $Cln3^{-/-}$ mice, interneuron loss was detected in the entorhinal cortex LII and LIV at 7 months (Mitchison et al., 1999) and in the hippocampus at 14 but not 5 months (Pontikis et al., 2004). In the $Cln3^{\Delta7/8}$ mouse, significant decreases in neuron numbers were determined in select thalamic nuclei (VPM/VPL) and in the somatosensory cortex at 12 months (Pontikis et al., 2005). Visual loss is a late occurrence in the JNCL mouse models (Cotman et al., 2002; Katz et al., 2008). The retinal architecture is preserved (Seigel et al., 2002; Weimer et al., 2006) and ERGs are normal at 11 months (Seigel et al., 2002). Microscopic examination indicates significant reduction in axon density (Sappington et al., 2003) or axon number (Katz et al., 2008) in the optic nerve, and reduced inner nuclear layer neuron numbers at 12 months (Katz et al., 2008). One study examined retino-targeted areas at 6 month, and determined 25% loss of projection neurons in the thalamic LGNd (Weimer et al., 2006).

Progressive motor and behavioral deficits have been documented in the JNCL mouse models. Decreased rotarod performance begins at 2 months in our *Cln3*[LacZ/LacZ] mice (Eliason et al., 2007), and has similarly been reported at 8 weeks in $Cln3^{\Delta7/8}$ mice (Osório et al., 2009). $Cln3^{\Delta7/8}$ mice display decreased exploratory behavior and ataxic gait at 8 weeks (Osório et al., 2009), and impairment in associative learning at 3.5 months (Wendt et al., 2005). In addition, our *Cln3*[LacZ/LacZ] mice display a clasping phenotype at 4 months (40% of the mice clasp), significant tremor at 5 months, and reduced nocturnal activity at 10 months (Eliason et al., 2007). Although spontaneous seizure activity has not been observed in JNCL mice, we found that our *Cln3*[LacZ/LacZ] mice exhibit enhanced susceptibility to drug induced seizures at 3 months of age (Eliason et al., 2007).

Recent studies implicate CLN3 in nervous system development. In the cerebellum of 1-week old $Cln3^{-/-}$ mice the internal granule cell layer thickness is reduced, and sporadic areas of Bergman gliosis are found in association with Purkinje cell loss (Weimer et al., 2009). Moreover, rotarod performance was found to be impaired at 2 weeks of age for these mice, A separate study shows delays in neonatal reflex milestones in $Cln3^{\Delta7/8}$ mice (Osório et al., 2009). Thus CLN3 appears to play a role in NS development in addition to maintenance of brain functions in mature mice.

To better understand the functions of CLN3 and the pathogenesis of JNCL in the nervous system, especially in central nervous system (CNS), reliable and detailed information is necessarily needed about the spatial and temporal localization of CLN3-expressing neurons in the nervous system. This information will enable us to know when and where in the nervous system CLN3 plays its roles and thus will guide us to identify correct targets for further investigation and for establishment of cellular models. Unfortunately, tissue localization of CLN3-expressing neurons appears very difficult due to low endogenous CLN3 levels and lack of suitable antibodies to CLN3. Some studies have used antibodies produced against a specific portion of CLN3 sequence to localize CLN3 in brain tissues, but information has been limited and inconsistent (Chattopadhyay and Pearce, 2000; Cotman et al., 2002; Ezaki et al., 2003; Luiro et al., 2001; Margraf et al., 1999; Pane et al., 1999).

Our reporter mouse serves as a sensitive tool to provide information regarding CLN3 expression patterns. In our preliminary study using heterozygous *Cln3*[LacZ/+] reporter mice, we observed CLN3 reporter expression in neurons of some brain regions such as retrosplenial cortex (RSC), dentate gyrus (DG), subiculum and entorhinal cortex (EC) during development, as well as in adult DG and retina (Eliason et al., 2007). In the present study, using both *Cln3*[LacZ/+] and *Cln3*[LacZ/LacZ] mice, a comparison of the cell density and staining intensity of CLN3 reporter neurons is made between these two types of mice derived from the same litters. More importantly, we have documented detailed temporal and spatial localization of CLN3 reporter neurons in the nervous system, contributing toward the understanding of the pathogenesis of JNCL.

Materials and methods

Generation of Cln3[lacZ/+] and Cln3[lacZ/lacZ] reporter mice

All animal procedures were approved by the University of Iowa Animal Care and Use Committee and conform to NIH guidelines. Transgenic mice were generated recently in this laboratory by knocking-in DNA sequence encoding nuclear-targeted bacterial ß-gal via homologous recombination of a targeting construct into embryonic stem cells such that ß-gal transcription is controlled by native sequence 5' to the CLN3 coding region, replacing most of exon 1 and all of exons 2–8, creating a null mutation (Eliason et al., 2007). Mice were backcrossed to C57BL/6 J and animals used in this study had undergone at least eight generations of backcrossing. Genotyping of the mice was done as previously described (Eliason et al., 2007). *Cln3*^{+/+}, *Cln3*[LacZ/+] and *Cln3*[LacZ/LacZ] mice at embryonic day 15 (E15) and E17 and at postnatal day 0 (P0, birthday), P1, P3, P4, P7, P10, P14, P21, P28, P30, P40, P60, P90, P120 and P240 (2–4 mice/each age) were used in the present study.

X-gal histochemistry

The prenatal mice were immerse-fixed in ice-cold 2% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.2) overnight. The postnatal mice were anesthetized with ketamine and xylazine and were perfused via heart with the PFA. Brains and cervical and thoracic spinal cords with dorsal root ganglions were dissected out and postfixed in the same fixative for 2 h at room temperature. Both prenatal mice and the nervous tissues from postnatal mice were put into 30% sucrose/PBS at 4 °C until they sank, and embedded in OCT (Tissue-Tek; Sakura Finechemical, Tokyo, Japan). Ten micrometer sequential sections were cut with a cryostat and thaw-mounted on glass slides. Slices were dried at room temperature and then stored at −80 °C until staining. For X-gal staining, selected slide sections were thawed and rinsed, and stained overnight with 5-bromo-4-chloro-3-indolyl ß-D-gal (X-gal; Gold Biotechnology, St. Louis, MO) solution at 37 °C. After rinsing, sections were counterstained with Neutral Red (0.15%) or Nuclear Red (0.1%). Sections were air dried for several hours and then coverslipped with Permount for analysis.