The neuronal ceroid lipofuscinosis \textit{Cln8} gene expression is developmentally regulated in mouse brain and up-regulated in the hippocampal kindling model of epilepsy

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Abstract

\textbf{Background:} The neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative disorders characterized by accumulation of autofluorescent material in many tissues, especially in neurons. Mutations in the \textit{CLN8} gene, encoding an endoplasmic reticulum (ER) transmembrane protein of unknown function, underlie NCL phenotypes in humans and mice. The human phenotype is characterized by epilepsy, progressive psychomotor deterioration and visual loss, while motor neuron degeneration (\textit{mnd}) mice with a \textit{Cln8} mutation show progressive motor neuron dysfunction and retinal degeneration.

\textbf{Results:} We investigated spatial and temporal expression of \textit{Cln8} messenger ribonucleic acid (mRNA) using \textit{in situ} hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and northern blotting. \textit{Cln8} is ubiquitously expressed at low levels in embryonic and adult tissues. In prenatal embryos \textit{Cln8} is most prominently expressed in the developing gastrointestinal tract, dorsal root ganglia (DRG) and brain. In postnatal brain the highest expression is in the cortex and hippocampus. Expression of \textit{Cln8} mRNA in the central nervous system (CNS) was also analyzed in the hippocampal electrical kindling model of epilepsy, in which \textit{Cln8} expression was rapidly up-regulated in hippocampal pyramidal and granular neurons.

\textbf{Conclusion:} Expression of \textit{Cln8} in the developing and mature brain suggests roles for \textit{Cln8} in maturation, differentiation and supporting the survival of different neuronal populations. The relevance of \textit{Cln8} up-regulation in hippocampal neurons of kindled mice should be further explored.

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\textbf{Background}

The neuronal ceroid lipofuscinoses (NCLs) comprise a group of human neurodegenerative disorders (CLN1-CLN8) characterized by epilepsy, visual failure,
psychomotor deterioration and accumulation of autofluorescent lipopigment in many tissues, especially in neurons [1]. Six genes underlying human NCLs have been identified and the proteins initially characterized (reviewed in [2]). Naturally occurring mouse models exist for CLN6 and CLN8 [3-5], while mouse models for CLN1, CLN2 and CLN3 have been generated by gene targeting [6-10].

The ubiquitously expressed CLN8 gene encodes a transmembrane protein which localizes to the ER and the ER-Golgi intermediate compartment in non-neuronal cells and to the ER in neuronal cells [4,11,12]. Mutations in CLN8 result in two distinct NCL phenotypes in humans: Northern epilepsy (Progressive epilepsy with mental retardation, EPMR, OMIM 600143) described in Finnish patients, and variant late infantile onset NCL in a subset of Turkish patients [4,13]. EPMR is characterized by frequent drug-resistant epileptic seizures with onset at 5–10 years of age, followed by progressive mental retardation [14], while the Turkish patients show earlier onset and a more rapid progression [15]. In EPMR, intracellular storage material, including subunit c of the mitochondrial ATP synthase, neuronal loss and neuronophagy [16].

We then estimated the level of Cln8 expression using real-time quantitative RT-PCR analysis. Cln8 showed highest expression in liver, spleen, heart and skeletal muscle, 2.5, 2.2, 1.7 and 1.6 fold higher (respectively) than in the brain (Fig. 1B). Expression was lowest in 11-day embryo and adult kidney, lung and testis, 0.6, 0.9, 0.5 and 0.03 fold lower than in the brain (Fig. 1B). This indicates that Cln8 expression levels in adult mouse tissues as well as in whole mouse embryos are not dramatically different.

**Cln8 is expressed throughout mouse development**

The tissue expression of Cln8 during development and brain maturation was characterized using radioactive mRNA in situ hybridization analysis. For this purpose, whole embryo sections from E13, E15.5 and E17 mice and brain sections from postnatal (P) P0, P5, P10 and adult mice were used and the expression of Cln8 was quantitated by a computer based MCID image analysis system.

In fresh-frozen E13 and E17 embryos the overall expression of Cln8 was weak. In E17 the most prominent expression was detected in the developing gastrointestinal tract (Fig. 2A, 2B). In addition, there was high expression in the DRG neurons (Fig. 2C, 2D). Some Cln8-specific signal was also detected in the developing brain (Fig. 2E). The expression of Cln8 in E13 was similar to that in E17 except in the DRG neurons, where no signal was detected (data not shown). In paraffin-embedded E15.5 embryos a Cln8-specific signal was detected in the developing gastrointestinal tract, adrenal glands and the developing brain, especially in the cortical plate (data not shown).

In postnatal mice (P0, P5, P10 and adult) Cln8 expression was quantitated in the cortex, cerebellum and different hippocampal regions. Expression at P0 was low but specific in hippocampal regions CA1, CA3 and the granular cell layer of the dentate gyrus and in the cortex (Fig. 3). At P5 and P10, the Cln8 expression increased in the hippocampal CA1 region (to 115% and 106% of P0) and especially in the CA3 region (to 139% of P0) (Fig. 3). In adult brain the expression levels of Cln8 were lower than at P0,
Northern blot and RT-PCR analysis of Cln8 mRNA expression in mouse tissues

**A**

Northern blot analysis of Cln8 transcripts in mouse tissues. A β-actin probe was used as a control. Molecular weight marker in kb is shown on the left.

**B**

Real-time quantitative RT-PCR analysis of Cln8 in mouse tissues. The expression of Cln8 in brain, given a value of 1, was used as a control. The expression of Cln8 in the other tissues is shown as a fold of the control.

**Figure 1**

**Northern blot and RT-PCR analysis of Cln8 mRNA expression in mouse tissues**

A: Northern blot analysis of Cln8 transcripts in mouse tissues. A β-actin probe was used as a control. Molecular weight marker in kb is shown on the left. B: Real-time quantitative RT-PCR analysis of Cln8 in mouse tissues. The expression of Cln8 in brain, given a value of 1, was used as a control. The expression of Cln8 in the other tissues is shown as a fold of the control.
In situ hybridization analysis of Cln8 mRNA expression in mouse embryos. In the E17 mouse embryo, developing gastrointestinal tract (A, B) and DRG (C, D), hybridized with Cln8 specific cRNA probe, are shown. Bright-field view of the cells is shown on the left (A, C) and dark-field detection of hybridization signals on the right (B, D). Optical density values of hybridization signals of Cln8 sense and antisense probes in E13 and E17 gut and brain tissues are shown in the diagram (E). Error bars represent standard error of the mean. s = sense, as = antisense
Figure 3
Differential distribution of Cln8 mRNA in brains of P0, P5, P10 and adult mouse. Dark-field emulsion autoradiographs from frontal sections, shown on the left, were analyzed by in situ hybridization analysis (A-D). Higher magnification in the hippocampal area CA3 is shown on the right. Bright-field views of the cells (a-d) and dark-field emulsion autoradiographs (a’-d’) are shown. Optical density values of hybridization signals of Cln8 antisense probe are shown as bars in the diagram below (A = adult). Optical density values of hybridization signals of Cln8 sense probe are shown as a gray area behind the bars. In each brain region the optical density values at P0, P5 and P10 were compared to the optical density value of adult. Error bars represent standard error of the mean. Symbols *, ** and *** represent p < 0.05, 0.01 and 0.001, respectively. CX = cortex, DG = dentate gyrus.
P5 and P10 in every region analyzed (Fig. 3). When compared with adults, a statistically significant difference in Cln8 expression was detected in the cortex at P0, P5 and P10 and in the CA3 region at P10 (Fig. 3). Expression of Cln8 in adult cerebellum was 156% of expression in the cortex (data not shown). In all maturation stages, the expression of Cln8 was highest in hippocampal region CA3 (Fig. 3).

**Cln8 expression is strongly up-regulated in the hippocampal electrical kindling model of epilepsy**

Epileptic seizures are a characteristic feature in human patients with CLN8 mutations. We therefore analyzed, by mRNA in situ hybridization analysis, possible changes in Cln8 expression 2 h, 6 h and 24 h after kindling-induced epileptic seizures. Animals were subjected to 40 rapid kindling stimulations which lead to increased excitability in mice at 4 weeks after the stimulation [25]. Each rapid kindling stimulation induced relatively short focal (grade 0–2) or long-lasting generalized (grade 4–5) seizures. The mean duration of afterdischarges was 20 ± 3 s for focal seizures and 76 ± 8 s for generalized seizures. All animals experienced multiple (11 ± 2) grade 4–5 generalized tonic-clonic seizures. The expression of Cln8 in the control, electrode implanted but non-stimulated mice (0 h; Fig. 4) was essentially identical to that in wild-type adult mouse brain (Fig. 3). After kindling stimulations Cln8 was strongly up-regulated in the hippocampus, especially in the CA3 region and most prominently in the granular cell layer of the dentate gyrus (Fig. 4). After 2 h, 6 h and 24 h expression of Cln8 in CA3 was 92%, 114% and 134% of that of controls, and in the granular cell layer of the dentate gyrus 128%, 225% and 286% of controls, respectively (Fig. 4). When compared to controls, a statistically significant increase in Cln8 expression was detected in the cortex after 6 h, and in the CA1 and CA3 regions, and the granular cell layer of the dentate gyrus after 2 h, 6 h and 24 h (Fig. 4). All changes in Cln8 mRNA expression after rapid kindling stimulations were bilateral with no differences between the sides.

**Discussion**

We analyzed the expression of the Cln8 gene during mouse development and in the hippocampal kindling model of epilepsy to gain understanding of the role of CLN8 in the disease mechanisms underlying both human and mouse NCLs. In northern blot analysis a Cln8 transcript of approximately 3 kb was detected in all tissues studied. Two additional transcripts, approximately 2 kb and 7 kb, were detected in several tissues. Thus, Cln8 expression resembles that of human CLN8, which is ubiquitously expressed with transcripts of 1.4 kb, 3.4 kb and 7.5 kb [4]. The open reading frame of mouse Cln8 is 82% identical with CLN8 and at the amino acid level the proteins are 85% identical [4]. In both human and mouse genes the RT-PCR analysis of the CLN8 open reading frame resulted in a single fragment, suggesting that the different size transcripts seen in northern analysis are due to alternative 3’ untranslated regions as previously suggested for the human CLN8 gene [4]. It is, however, also possible that the CLN8/Cln8 genes have several transcription initiation sites. Real-time quantitative RT-PCR analysis further suggested that the expression levels of Cln8 are relatively low in both adult and embryonic tissues, and showed that expression levels in various tissues do not differ dramatically. The remarkably low expression of Cln8 in testes could reflect a high level of the control gene analyzed in this tissue. Despite ubiquitous expression of the underlying gene and accumulation of storage material in all tissues, the most severe damage in EPMR patients and mnd mice is in neuronal cells. This is also characteristic of other NCLs, suggesting that NCL proteins may have a specific function in neuronal cells distinct from the role in non-neuronal cells, or that certain neuronal populations may be more sensitive to the disturbed function of NCL proteins.

In situ hybridization analysis of developing mice indicates that Cln8 mRNA is expressed throughout development as well as in the mature brain. The expression of Cln8 in embryonic stages E13 and E17 was specific but low in many tissues including brain, but remarkably high in the developing gastrointestinal tract. This may indicate a role for Cln8 in the innervation of the developing gut. In addition, in the E17 embryo Cln8 expression was high in DRG neurons. These neurons determine the connections between interneurons and motoneurons of the spinal cord. Assuming a role for Cln8 in this process, its disturbed function might result in defective connections in DRG with consecutive dysfunction of motor neurons resulting in progressive paralysis in the mnd mouse model [5,17]. The expression of Cln8 in postnatal mouse brain was highest at developmental stages P5 and P10, especially in hippocampal region CA3. The brain expression was clearly lower in adult mice than at P0, P5 and P10, suggesting a role for Cln8 in brain maturation. This early postnatal period is characterized by a rapid increase in the number of synapses, as well as maturation of oligodendrocytes, the myelin-forming cells in the CNS [26]. Two other NCL proteins, Ppt1 and Cln3, have been localized to synaptic areas of neurons [27,28] suggesting the importance of these NCL proteins for synaptic functions. However, detailed study of the function of Cln8 in the CNS and different brain cells is currently hampered by the lack of a specific antibody detecting endogenous Cln8. Very low levels of Cln8 mRNA were also detected in developing and adult motoneurons and retina (data not shown). However, the Cln8 transcript levels in these samples were virtually indistinguishable from the respective sense controls.
Figure 4
Distribution of Cln8 mRNA in brains of hippocampal kindling induced epileptic mice. Frontal mouse brain sections were analyzed 2 h, 6 h and 24 h after kindling induced epileptic seizures by in situ hybridization. Mice with electrodes implanted but without electrical stimulations were used as controls (0 h). Dark-field emulsion autoradiographs of hippocampal Cln8 expression in kindling-induced mice 0 h and 24 h after kindling are shown. Optical density values of hybridization signals of Cln8 antisense probe are shown as bars in the diagram. Optical density values of hybridization signals of Cln8 sense probe are shown as a gray area behind the bars. In each brain region the optical density values 2 h, 6 h and 24 h after kindling-induced seizures were compared to controls (0 h). Error bars represent standard error of the mean. Symbols *, ** and *** represent p < 0.05, 0.01 and 0.001, respectively. DG = dentate gyrus
The expression of several other NCL genes in mouse and/or rat has been characterized. In addition to Cln8, Cln1 and Cln5 also show developmental regulation, whereas the expression of two other NCL genes, cathepsin D underlying a naturally occuring sheep NCL model and Cln2 underlying a human late infantile-onset NCL form, is relatively constant in the rat brain during development [29-32]. Mouse Cln3 is expressed in adult mice throughout the brain, and in the hippocampus the most prominent expression is detected in the granular cell layer of the dentate gyrus and pyramidal cells [28]. In addition, human CLN1 and CLN5 genes are expressed at the beginning of neurogenesis in embryonic human brains [33]. This expression increases as brain development proceeds. Expression of NCL genes, including Cln8, in the developing and mature brain indicates that NCL proteins may have roles not only in supporting the survival of neurons but also in the maturation and differentiation of different neuronal populations during development. Defects in maturation processes could potentially lead to neuronal degeneration and loss of neurons.

In the kindling model of experimental epilepsy a rapid up-regulation of Cln8 expression was detected in the brain, especially in hippocampal regions CA3 and the granular cell layer of the dentate gyrus. The expression of Cln8 increased at every time point measured, and was highest 24 h after kindling. Whether single or focal seizures alone are sufficient to induce Cln8 expression requires further investigation. While only Cln8 expression has been investigated in the kindling model, expression of cathepsin D and the Cln1 encoded palmitoyl protein thioesterase 1 (Ppt1) protein has been studied using kainic acid-induced seizures in rats as a model. Both show increased expression in this model, cathepsin D in the hippocampus, limbic cortex and temporo-parieto-occipital neocortex [34] and the Ppt1 protein most prominently in pyramidal cells of hippocampal regions CA1 and CA3 [35]. Although different forms of NCLs are genetically heterogeneous, they share a common hippocampal pathology, which is distinct from lesions caused by anoxic-ischemic events and is instead suggested to be a consequence of primary metabolic defects [36]. Increased plasma glutamate levels, decreased glutamate uptake, decreased glutamate transporters as well as changes in ionotropic glutamate receptors has been described in mnd mice [37,38]. Also, prominent loss of GABAergic interneurons in mnd as well as in the Cln1 and Cln3 knock-out mice has been reported [39-41]. Alterations in both glutamatergic and GABAergic neurotransmission may both contribute to chronic excitotoxicity, hypothesized to underlie the cellular dysfunction and brain pathology in NCL disorders [42].

Conclusion

The Cln8 gene is widely expressed in embryonic and adult mouse tissues. In line with this, and comparable with other NCL disorders, accumulation of storage deposits in CLN8-associated disorders occurs in all tissues. However, as the most striking accumulation and cellular dysfunction is limited to neurons, it is likely that CLN8/Cln8 is involved in a neuron-specific biochemical pathway or that neuronal cells are more sensitive to accumulating material or lack compensatory pathways.

The Cln8 gene is expressed throughout brain development and also in mature brain, suggesting a role for Cln8 in maturation and differentiation of neurons and in supporting the survival of neurons. The expression of Cln8 showed regional differences, suggesting a specific function for Cln8 in specific neuronal populations. The relevance of Cln8 up-regulation in hippocampal neurons of kindled mice needs to be studied further.

Methods

Northern blot analysis

FirstChoice™ Northern Blot Mouse Blot I (Ambion, Austin, TX, USA) was used to analyze the distribution of Cln8 expression in various tissues. An 872 base pair (bp) deoxyribonucleic acid (DNA) fragment including a Cln8 open reading frame of 867 bp, digested from the SvPoly-Cln8 expression vector with restriction enzyme BamHI, was used as a probe. The hybridization was performed according to the manufacturer's instructions with Ultra sensitive Hybridization buffer (Ambion), and salmon sperm DNA and Cot-1 DNA for blocking.

Real-time quantitative RT-PCR analysis of Cln8

Real-time quantitative RT-PCR analysis of Cln8 was performed in an ABI PRISM® 7000 SDS thermal cycler (Applied Biosystems, Foster City, CA, USA) using 5 µl of each tissue from Mouse Multiple Tissue cDNA (MTC™) Panel II (BD Biosciences) as template. Each 25 µl reaction contained 300 nM of reverse primer 5'-CCACTGGTT-GGCTTTCCA-3' and 900 nM of forward primer 5'-GCCCTTCACGTGATTTCC-3' as well as 200 nM of Cln8 specific probe 6FAM-TGACCACCCAGCTTCAAACAT-TAMRA in TaqMan® Universal PCR Master Mix (Applied Biosystems). The primers were optimized for analysis and the PCR fragment of 76 bp covered the region from 510 to 585 bp of the Cln8 open reading frame (GenBank sequence accession no AF125307). The Cln8 specific probe included a reporter dye 6-carboxyfluorescein (FAM) and a quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Endogenous control reactions were performed using Assays-on-Demand Mm00446973_m1 TATA-box binding protein amplification according to the manufacturer's instructions (Applied Biosystems). Each reaction was performed in triplicate. Standard complementary
DNA (cDNA) solutions, generated from C57/BL mice cortex mRNA, and standard curve method were used ([User Bulletin #2: ABI PRISM(R) 7700 Sequence Detection System. P/N 4308359B (2001) Applied Biosystems. 36 s]). Statistical analyses were performed using Microsoft Excel.

The DNA fragment covering the whole open reading frame of \textit{Cln8} was PCR amplified using primers 3'-GTGATTTCCTCCGGTGCTAGG-5' and 3'-GCAACCACCATTTTCCTAGG-5'.

\textbf{In situ hybridization}

Paraffin and cryosections of NMRI or C57/BL mice were prepared and analyzed by mRNA \textit{in situ} hybridization as previously described [43-45] with slight modifications. The isolated tissues from postnatal wild-type and kindling mice were fresh-frozen and sectioned. Embryos were either fixed in 4% paraformaldehyde and embedded in paraffin or fresh-frozen and sectioned. For all serial sections, 8–14 µm, of 35S-labeled 370 bp \textit{Cln8} specific complementary RNA (cRNA) probe (from bp 29 to 398; GenBank AF125307) was used. For the generation of the antisense and control sense cRNAs, the vector (pBluescript vector; Stratagene, La Jolla, CA, USA) containing the PCR-amplified \textit{Cln8} fragment was linearized and used as a template for RNA polymerases T3 and T7, respectively, using α-35S-labeled UTP (Amersham Biosciences, Uppsala, Sweden).

The paraffin sections were treated as cryosections, after first being deparaffinized in xylene and rehydrated in a descending ethanol series. All the slides were fixed in 4% paraformaldehyde, rinsed twice in phosphate-buffered saline and treated with proteinase K (20 µg/ml for paraffin sections, 1 µg/ml for cryosections). Next, the slides were postfixed in 4% paraformaldehyde, and the cryosections rinsed in 50% deionized formamide in 2× standard sodium citrate (SSC). After being washed in distilled water, all slides were acetylated for 10 minutes with 0.25% acetic anhydride in 0.1 M triethanolamine. The paraffin sections were then dehydrated in an ascending ethanol series, while the cryosections were rinsed again with 50% formamide in 2× SSC. Prehybridization for 1 h was subsequently performed at +52°C with hybridization buffer containing 60% formamide, 10% dextran sulphate, 1× Denhardt’s solution, 0.5 µg/ml yeast transfer RNA (tRNA), and 100 mM dithiothreitol. After prehybridization, cRNA probes were added to the hybridization buffer at a concentration of 35 000 cpm/µl, and hybridization was carried out overnight at +52°C in a chamber humidified with 60% formamide and 5× SSC.

After hybridization the sections were first washed in 5× SSC, 10 mM dithiothreitol at +50°C for 30 minutes, followed by 30 minutes in 50% formamide, 2× SSC, 30 mM dithiothreitol at +65°C, and 3× 10 minutes in NTE-buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA) at +37°C. Next, the slides were treated with RNase A (20 µg/ml) in NTE-buffer at +37°C for 30 min and washed for 15 min in NTE-buffer at +37°C. Washing with 50% formamide, 2× SSC, 30 mM dithiothreitol was then repeated, after which the sections were washed in 2× SSC and 0.1× SSC, each for 15 min at +37°C. The slides were dehydrated at room temperature with ethanol containing 0.3 M ammonium acetate, air-dried, and exposed to BioMax MR Film (Kodak) together with 14C radioactive standards (Amersham Biosciences) for 5 days. Finally, the sections were dipped in NTB-2 emulsion (Kodak), developed after 3 to 4 weeks using D-19 developer (Kodak), fixed with Kodak Unifix, counterstained with hematoxylin (Shandon Inc./Thermoshandon, Pittsburgh, PA, USA), and mounted with Permount (Fisher Scientific International Inc., Hampton, NH, USA). Mice were bred in the Viikki Laboratory Animal Center, University of Helsinki. The experiments were conducted according to the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purposes" with the approval of the institutional ethics committee.

\textbf{Rapid kindling procedure}

Adult male C57/BL mice weighing 22–25 g (n = 16) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), and bipolar stainless steel electrodes (Plastics One) were implanted in the left ventral hippocampus using a Kopf stereotaxic frame as previously described [46]. One week later, the mice were electrically stimulated with forty threshold stimulations with 5 min intervals (10 Hz frequency, 1 ms square-wave pulses for 10 s). The electroencephalogram was continuously recorded during the whole rapid kindling procedure using the MacLab/4e system (ADInstruments). To determine the threshold, current intensity was increased stepwise (10 µA increments, starting from 10 µA) every 5 min until focal epileptiform activity (afterdischarge) of at least 5 s duration was elicited. Rapid kindling stimulations were then given on the same day. Behavioral convulsions were scored according to a modification [25] of the scale of Racine [47]: grade 0, arrest, normal behavior; grade 1, facial twitches; grade 2, chewing and nodding; grade 3, forelimb clonus; grade 4, rearing, body jerks, tail upholding; and grade 5, imbalance, hind-limb clonus and vocalization. At 2, 6 and 24 h after the last stimulus-evoked seizure, animals were decapitated (four animals in each group) and brains were processed for \textit{in situ} hybridization. Four non-stimulated, electrode-implanted mice served as controls and were killed together with the experimental animals from different groups. The correct localization of the electrode was controlled for each animal in the coronal sections of the brain used for \textit{in situ} hybridization. All experimental pro-
cedures were approved by the Research Ethics Committee at the Medical Faculty of the University of Lund.

Quantitative and statistical analysis

Quantification of radioactive in situ hybridization films was done by digitizing the film images with a computer-based MCID image analysis system (Imaging Research). Gray levels from 14C radioactive standards were used in a third-degree polynomial calibration to obtain equivalent values of tissue radioactivity (nCi/g) for different brain regions. Measurements for each structure were carried out in several sections per animal. Since there were no significant differences between the two sides, values were pooled to obtain the mean value for each animal and brain region. Data from four control and four experimental animals for every probe and time point were used for analysis. Statistical analysis was performed using Student's t-tests. In the figures, the densities of hybridization signals of sense and antisense probes are shown separately. Sense values were not subtracted from antisense values because of different probes used. Error bars represent standard error of the mean; symbols *, ** and *** represent p < 0.05, 0.01 and 0.001, respectively.

Authors' contributions

LL carried out real-time quantitative PCR and northern blot analyses. In addition, LL was involved in the analysis of mRNA in situ results and design of the study, drafted the manuscript and made the figures. AA carried out the mRNA in situ analysis and was involved in drafting the manuscript. OK prepared several mouse tissue samples for the mRNA in situ analysis, was involved in the analysis of the mRNA in situ results, and revised the manuscript. MK established the real-time quantitative RT-PCR methodology and helped with the analysis. ZK provided all kindling samples and contributed significantly to figures 3 and 4. mRNA in situ analyses were conducted under the supervision of MS, who also revised the manuscript and was involved in the design of the study. AEL was involved in the design of the study, analysis of the results and revision of the manuscript.

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References

1. Santavuori P: Neuronal ceroid-lipofuscinoses in childhood. Brain Dev 1988, 10:80-83.
2. Halila M: The neuronal ceroid-lipofuscinoses. J Neuropathol Exp Neuro 2003, 62:1-13.
3. Bronson RT, Donahue LR, Johnson KR, Tanner A, Lane PW, Faust JR: Neuronal ceroid lipofuscinosis (nclf), a new disorder of the mouse linked to chromosome 9. Am J Med Genet 1998, 77:289-297.
4. Ranta S, Zhang Y, Ross B, Lonka L, Takkunen E, Messer A, Sharp J, Wheeler R, Kusumi K, Mole S, Liu W, Soares MB, Bonafolino MF, Hirvsniami A, de la Chapelle A, Gilliam TC, Lehesjoki AE. The neuronal ceroid lipofuscinoses in human EMPR and mnd mutant mice are associated with mutations in CLN8. Nat Genet 1999, 22:323-326.
5. Messer A, Flaherty L: Autosomal dominance in a late-onset motor neuron disease in the mouse. J Neurogenet 1986, 3:345-355.
6. Gupta P, Soyombo AA, Azahsband A, Wisniewski KE, Shelton JM, Richardson JA, Hamner RE, Hofmann SL: Disruption of PPT1 or PPT2 causes neuronal ceroid lipofuscinosis in knockout mice. Proc Natl Acad Sci USA 2001, 98:13566-13571.
7. Katz ML, Shibuya H, Liu PC, Kaur S, Gao CL, Johnson GS: A mouse gene knockout model for juvenile ceroid-lipofuscinosis (Batten disease). J Neurosci Res 1999, 57:551-556.
8. Mitchison HM, Bernard DJ, Greene ND, Cooper JD, Junaid MA, Pullarakt RK, de Vos N, Breuning MH, Owens JW, Mobley WC, Gardner RM, Lake BD, Taschner PE, Nussbaum RL: Targeted disruption of the Cln3 gene provides a mouse model for Batten disease. The Batten Mouse Model Consortium [corrected]. Neurobiol Dis 1999, 6:321-334.
9. Cotman SL, Vrabanc V, Lebl LA, Lee RL, Johnson KA, Donahue LR, Teed AM, Antonellis K, Bronson RT, Lerner TJ, MacDonald ME: Cln3(Deltaex7/8) knock-in mice with the common CLN3 mutation exhibit progressive neurologic disease that begins before birth. Hum Mol Genet 2002, 11:2709-2721.
10. Koike M, Nakashiba H, Safig P, Etsuki I, Ishikawa K, Ohawa Y, Schulz-Schaeffer W, Watanabe T, Waguri S, Kametaka S, Shibata M, Yamanoto K, Kominami E, Peters C, von Figure K, Uchiyama Y: Cathepsin D deficiency induces lysosomal storage with cerebral lipofuscin in mouse CNS neurons. J Neurosci 2000, 20:6998-6906.
11. Lonka L, Kytälä A, Ranta S, Jalkano A, Lehesjoki AE: The neuronal ceroid lipofuscinosis CLN8 membrane protein is a resident of the endoplasmic reticulum. Hum Mol Genet 2000, 9:1691-1697.
12. Lonka L, Salonen T, Siintola E, Kopra O, Lehesjoki AE, Jalkano A: Localization of wild-type and mutant neuronal ceroid lipofuscinosis CLN8 proteins in non-neuronal and neuronal cells. J Neurosci Res 2004, 76:862-871.
13. Ranta S, Topcu M, Tegelberg S, Tan H, Ustebutun A, Saatchi I, Dufke A, Enders H, Pohl K, Alenink Y, Mitchell WA, Molle SE, Lehesjoki AE: Variant late infantile neuronal ceroid lipofuscinosis in a subset of Turkish patients is allelic to Northern epilepsy. Hum Mutat 2004, 23:300-305.
14. Hirvsniami A, Lang H, Lehesjoki AE, Leisti J: Northern epilepsy syndrome: an inherited childhood onset epilepsy with associated mental deterioration. J Med Genet 1994, 31:177-182.
15. Topcu M, Tan H, Yaliizgili D, Ustebutun A, Saatchi I, Ayanci M, Anlar B, Topaloglu H, Turanli G, Kose G, Aysun S: Evaluation of 36 patients from Turkey with neuronal ceroid lipofuscinosis: clinical, neurophysiological, neuroradiological and histopathological studies. Turk J Pediatr 2004, 46:1-10.
16. Herva R, Tyyneila J, Hirvsniami A, Syrijakallio-Yllitalo M, Haltia M: Northern epilepsy: a novel form of neuronal ceroid-lipofuscinosis. Brain Pathol 2000, 10:215-222.
17. Messer A, Strominger NL, Mazurkiewicz J: Histopathology of the late-onset motor neuron degeneration (Mnd) mutant in the mouse. J Neurogenet 1987, 4:201-213.
18. Messer A, Plummer J, Wong V, Lavall MM: Retinal degeneration in motor neuron degeneration (mnd) mutant mice. Exp Eye Res 1993, 57:637-641.
19. Messer A, Plummer J: Accumulating autofluorescent material as a marker for early changes in the spinal cord of the Mnd mouse. Neuromuscul Disord 1993, 3:129-134.
20. Bronson RT, Lake BD, Cook S, Taylor S, Davissson MT: Motor neuron degeneration of mice is a model of neuronal ceroid lipofuscinosis (Batten's disease). Ann Neurol 1993, 33:381-385.

21. Callahan LM, Wylen EL, Messer A, Mazurkiewicz JE: Neurofilament distribution is altered in the Mnd (motor neuron degeneration) mouse. J Neuropathol Exp Neurol 1991, 50:491-504.

22. Mazurkiewicz JE, Callahan LM, Swash M, Martin J, Messer A: Cytoplasmic inclusions in spinal neurons of the motor neuron degeneration (Mnd) mouse. I. Light microscopic analysis. J Neurol Sci 1993, 116:59-66.

23. Goddard GV, McIntyre DC, Leech CK: A permanent change in brain function resulting from daily electrical stimulation. Exp Neurol 1969, 25:295-330.

24. Dennison Z, Teskey GC, Cain DP: Persistence of kindling: effect of partial kindling, retention interval, kindling site, and stimulation parameters. Epilepsia Res 1995, 21:171-182.

25. Nanobashvili A, Kokaia Z, Lindvall O: Generalization of rapidly recurring seizures is suppressed in mice lacking glial cell line-derived neurotrophic factor family receptor alpha2. Neuroscience 2003, 118:845-852.

26. Jacobson M: Developmental Neurobiology New York and London: Ple num Press; 1991.

27. Lehtovirta M, Kyttälä A, Eskelinen EL, Hess M, Heinonen O, Jalanko A: Palmitoyl protein thioesterase (PPT) localizes into synaptosomes and synaptic vesicles in neurons: implications for infantile neuronal ceroid lipofuscinosis (INCL). Hum Mol Genet 2001, 10:69-75.

28. Luiro K, Kopra O, Lehtovirta M, Jalanko A: CLN3 protein is targeted to neuronal synapses but excluded from synaptic vesicles: new clues to Batten disease. Hum Mol Genet 2001, 10:2123-2131.

29. Suopanki J, Tyynela J, Baumann M, Haltia M: The expression of palmitoyl-protein thioesterase is developmentally regulated in neural tissues but not in nonneural tissues. Mol Genet Metab 1999, 66:290-293.

30. Suopanki J, Tyynela J, Baumann M, Haltia M: Palmitoyl-protein thioesterase, an enzyme implicated in neurodegeneration, is localized in neurons and is developmentally regulated in rat brain. Neurosci Lett 1999, 265:53-56.

31. Isosomppi J, Heinonen O, Hiltunen JO, Greene ND, Vesa J, Uusitalo A, Mitchison HM, Saarma M, Jalanko A, Peltonen L: Developmental expression of palmitoyl protein thioesterase in normal mice. Brain Res Dev Brain Res 1999, 118:1-11.

32. Tyynela J, Sohar I, Sletat DE, Gin RM, Donnelly RJ, Baumann M, Haltia M, Lobel P: A mutation in the ovine cathepsin D gene causes a congenital lysosomal storage disease with profound neurodegeneration. Embo J 2000, 19:2786-2792.

33. Heinonen O, Salonen T, Jalanko A, Peltonen L, Copp A: CLN-1 and CLN-5, genes for infantile and variant late infantile neuronal ceroid lipofuscinosis, are expressed in the embryonic human brain. J Comp Neurol 2000, 426:406-412.

34. Herman M, Filipkowski RK, Domagala W, Kaczmarek L: Elevated cathepsin D expression in kainate-evoked rat brain neurodegeneration. Exp Neurol 1995, 136:53-63.

35. Suopanki J, Lintunen M, Lahtinen H, Haltia M, Panula P, Baumann M, Tyynela J: Status epilepticus induces changes in the expression and localization of endogenous palmitoyl-protein thioesterase 1. Neurobiol Dis 2002, 10:247-257.

36. Haltia M, Herva R, Suopanki J, Baumann M, Tyynela J: Hippocampal lesions in the neuronal ceroid lipofuscinoses. Eur J Paediatr Neurol 2001, 1(Suppl A):209-211.

37. Pennini T, Bastone A, Crespi D, Comolotti D, Manzoni C: Spinal cord GLT-1 glutamate transporter and blood glutamic acid concentrations in motor neuron degeneration (Mnd) mice. J Neurosci 1998, 18:31-36.

38. Pennini T, Bigi E, Ravizza T, Yezzani A, Calvaresi N, Tortarolo M, Biondetti C: Expression of glutamate receptor subtypes in the spinal cord of control and mnd mice, a model of motor neuron disorder. J Neurosci Res 2002, 70:553-560.

39. Cooper JD, Messer A, Feng AK, Chua-Couzens J, Moley WC: Apparent loss and hyper trophy of interneurons in a mouse model of neuronal ceroid lipofuscinosis: evidence for partial response to insul in-like growth factor-1 treatment. J Neurosci 1999, 19:2556-2567.

40. Bibie E, Gupta P, Hofmann SL, Cooper JD: Regional and cellular neuropathology in the palmitoyl protein thioesterase-1 null mutant mouse model of infantile neuronal ceroid lipofuscinosis. Neurobiol Dis 2004, 16:346-359.

41. Cooper JD: Progress towards understanding the neurobiology of Batten disease or neuronal ceroid lipofuscinosis. Curr Opin Neurobiol 2003, 13:21-28.

42. Das AM: Regulation of the mitochondrial ATP-synthase in health and disease. Mol Genet Metab 2003, 79:71-82.

43. Hiltunen JO, Arumae U, Moshnyakov M, Saarma M: Expression of mRNAs for neurotrophins and their receptors in developing rat heart. Circ Res 1996, 79:930-939.

44. Kokaia Z, Airaksinen MS, Nanobashvili A, Larsson E, Kujamaki E, Lindvall O, Saarma M: GDNF family ligands and receptors are differentially regulated after brain insults in the rat. Eur J Neurosci 1999, 11:1202-1216.

45. Lauren J, Airaksinen MS, Saarma M, Timmusk T: Two novel mammalian Nogo receptor homologs differentially expressed in the central and peripheral nervous systems. Mol Cell Neurosci 2003, 24:581-594.

46. Nanobashvili A, Airaksinen MS, Kokaia M, Rossi J, Azzetley F, Olosdorfer K, Mohapel P, Saarma M, Lindvall O, Kokaia Z: Development and persistence of kindling epilepsy are impaired in mice lacking glial cell line-derived neurotrophic factor family receptor alpha 2. Proc Natl Acad Sci U S A 2000, 97:12312-12317.

47. Racine RJ: Modification of seizure activity by electrical stimulation. I. Motor seizure. Electroencephalogr Clin Neurophysiol 1972, 32:281-294.