Early changes in gene expression in two models of Batten disease

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Abstract **Infantile and juvenile neuronal ceroid lipofuscinosis (NCLs) are progressive neurodegenerative disorders of childhood with distinct ages of clinical onset, but with a similar pathological outcome. Infantile and juvenile NCL are inherited in an autosomal recessive manner due to mutations in the *CLN1* and *CLN3* genes, respectively. Recently developed *Cln1*- and *Cln3*-knockout mouse models share similarities in pathology with the respective human disease. Using oligonucleotide arrays we identified reproducible changes in gene expression in the brains of both 10-week-old *Cln1*- and *Cln3*-knockout mice as compared to wild-type controls, and confirmed changes in levels of several of the cognate proteins by immunoblotting. Despite the similarities in pathology, the two mutations affect the expression of different, non-overlapping sets of genes. The possible significance of these changes and the pathological mechanisms underlying NCL diseases are discussed.**

Key words: Batten disease; Neuronal ceroid lipofuscinose; CLN1; Palmitoyl-protein thioesterase-1; CLN3; Microarray; Gene expression; Neurodegeneration

1. Introduction

Gene expression profiling in disease can furnish information on the pathogenesis of the disease at the molecular level and provide potential markers for disease progression. We report the use of high-density oligonucleotide arrays to examine the mRNA profile for approximately 36 000 genes in the brain of mouse models for two types of childhood neurodegenerative disorders: infantile and juvenile neuronal ceroid lipofuscinosis (NCL). Both infantile (INCL) and juvenile NCL (JNCL) are inherited in an autosomal recessive manner; JNCL represents the most common type of progressive neurodegenerative disease in children. JNCL is characterized initially by visual deterioration at age 5–7 years that ultimately results in blindness, followed by an increased frequency of seizures, mental retardation, loss of motor skills and premature death [1,2]. INCL has a similar course, but has onset before 2 years of age. The *CLN1* and *CLN3* genes were identified in 1995 [3,4]. The *CLN1* gene encodes a palmitoyl-protein thioesterase (PPT1) that is involved in the lysosomal metabolism of fatty acylated proteins [5]. The *CLN3* gene encodes a transmembrane protein of unknown function, most likely localized to the late endosome/lysosome in non-neuronal cell types and possibly in synaptic vesicles in neurons, reviewed in [6]. In both INCL and JNCL, autofluorescent hydrophobic material accumulates in the lysosomes of neurons and other cell types. However, the major component of the storage material is different in each disease type. In INCL the major component of the lysosomal storage material has been identified as saposins A and D [7]. Since saposins A and D are not PPT1 substrates, and because these proteins are also elevated in other lysosomal disorders, their accumulation may represent a thematic pathology resulting from lysosomal dysfunction. In JNCL, a predominant component of the lysosomal storage material has been identified as mitochondrial ATP synthase subunit c [8–11]. One of the paradoxes of NCL-disease is that the accumulation of this lysosomal storage material does not apparently lead to disease in non-neuronal cell types. Moreover, how these cellular alterations relate to the neurodegeneration in NCL-disease is unknown. Both the CLN1 and CLN3 proteins have been localized to late endosomes and lysosomes in non-neuronal cell types [12–14].

Cln1-knockout mice homozygous for a truncation of the *CLN1* gene product have been previously reported to have characteristic accumulation of autofluorescent material in the CNS and to have neurologic abnormalities [15]. *Cln3*-knockout mice homozygous for a targeted deletion of exons 1–6 in the *Cln3* gene have been previously reported to show characteristic accumulation of autofluorescent lipopigments containing mitochondrial ATP synthase subunit c in neural tissue, and selective loss of GABAergic neurons [16]. At 10 weeks of age both *Cln1*- and *Cln3*-knockout mice show evidence of storage material, but no reported evidence of behavioral or neurologic dysfunction. To explore changes in gene expression that precede these degenerative changes, we have used high-density oligonucleotide arrays to compare the expression of approximately 36 000 transcripts in the brain of 10-week-old *Cln1*-, *Cln3*-knockout and wild-type control mice. Reproducible, but distinct changes in expression were evident

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in both *Cln1*- and *Cln3*-knockout mice, as compared to normal, suggesting that there is a difference in the molecular mechanisms that underlie INCL and JNCL, respectively. This data-set provides an important illustration as to what may be occurring at the molecular level in the brain of homozygous *Cln1*- and *Cln3*-knockout mice that undergo neurodegeneration as a result of lacking functional CLN1 and CLN3 protein, respectively.

2. Materials and methods

2.1. Animals

Ten-week-old wild-type control C57BL6J/129S6/SvEv and homozygous *Cln1*-knockout mice on the same mixed background [15], and 10-week-old wild-type control 129S6/SvEv and homozygous *Cln3*-knockout mice on a 129S6/SvEv background [16] were used for this study. All procedures were carried out in accord with NIH guidelines and the University of Rochester Animal Care and Use Committee Guidelines.

2.2. Gene expression studies and data analysis

For comparative gene expression studies, whole brain from three 10-week-old wild-type control and *CLN3*-knockout mice (or similarly, wild-type and *CLN1*-knockout mice) were pooled, respectively, and homogenized by standard procedures in Trizol (Gibco BRL) for mRNA extraction. Total RNA from each sample was used to generate a high-fidelity cDNA, which is modified at the 5′-end to contain an initiation site for T7 RNA polymerase as per the manufacturer's protocol (SuperChoice, Gibco BRL). Upon completion of cDNA synthesis 1 μg of product was used in an in vitro transcription (IVT) reaction that contained biotinylated UTP and CTP, which are utilized for detection following hybridization to the microarray as per the manufacturers protocol (ENZO). Full-length IVT product (20 μg) was subsequently fragmented in 200 mM Tris–acetate (pH 8.1), 500 mM KOAc and 150 mM MgOAc at 94°C for 35 min. Following fragmentation all components generated throughout the processing procedure (cDNA, full-length cRNA, and fragmented cRNA) were analyzed by gel electrophoresis to assess the appropriate size distribution prior to microarray analysis.

Gene expression was analyzed using the Affymetrix U74 high-density oligonucleotide array set at the University of Rochester Microarray Core Facility. Each gene on the array is represented by 16–20 pairs of 25 mer oligonucleotides that span the coding region for each gene represented. Each probe pair consists of a perfect match sequence that is complementary to the cRNA target and a mismatch sequence that has a single base pair mutation in a region critical for target hybridization; this sequence serves as a control for non-specific hybridization. Hybridization, staining and washing of all arrays was performed in the Affymetrix fluidics module as per the manufacturer's protocol. Streptavidin phycoerythrin stain (Molecular Probes) was the fluorescent conjugate used to detect hybridized target sequences. Detection and quantification of target hybridization was performed with a GeneArray Scanner (Hewlett Packard/Affymetrix) set to scan each array twice at a factory set photomultiplier tube (PMT) level and resolution. All arrays were scanned pre- and post-antibody amplification with respect to the dynamic range of the scanner. Several genes have been identified on each array to help assess the overall quality of signal intensity from all arrays. The result of this analysis has demonstrated that all arrays are within a 0.2-fold difference of each other at baseline.

The Microarray Analysis Suite version 5 (Affymetrix) was employed to generate the comparative analysis presented in this study. Algorithms were used as described with thresholds set to default levels as defined by the manufacturer. All arrays within this data-set were normalized via global scaling (target intensity = 2500), and Super Scoring (SDT = 3) was applied to all probe sets of eight probe pairs or more, meaning that any probe pairs average difference that exceeded 3 standard deviations of the mean of all probe sets was not calculated in the average difference metric. All data represented are from the average of all pair-wise comparisons in the same direction.

2.3. RT-PCR

Amplification of a portion of each gene was performed using approximately 0.4 μg total RNA extract using a Life Technologies SuperScript One-Step RT-PCR system, according to the manufacturer's guidelines. Primers used for amplification were: GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; AI505387, 5′-GTTATTGGCTACACCAATTAAAAC-3′ and 5′-TGTTGTAAAACGCATGAGG-3′

2.4. Immunoblot analysis

Whole-brain extracts from 10-week-old wild-type control and CLN3 knockout mice were prepared in ice-cold 50 mM sodium phosphate buffer containing 0.5 mM dithiothreitol (DTT; pH 7.5) using pellet pestles. Protease inhibitor cocktail (Sigma, catalog No. P27140) was added to the samples to a final concentration of 2×. Following homogenization on ice, an equal volume of 50 mM sodium phosphate, 0.5 mM DTT, and 0.2% SDS (pH 7.5) was added and samples were centrifuged at 13 000 rpm for 5 min at 4°C to remove cellular debris. Proteins (10 μg) were separated on a 13% SDS–PAGE gel. Immunoblotting was performed using a primary COMT antibody at a 1:5000 dilution (BD Transduction) and the secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG) at 1:3500 dilution. Immunodetection was performed with an ECL-PLUS kit (Amersham-Pharmacia Biotech, Piscataway, NY, USA) as per the manufacturer's instructions. Similar procedures were used for immunoblotting of *Cln1*-knockout mice with the exception that 50 μg of protein was loaded per lane and immunoblotting was performed using a commercially (Promega, Cat. No. G5601) available antibody directed against glial fibrillary acid protein (GFAP) or with a mouse monoclonal antibody directed against platelet activating factor acetylhydrolase-2 (PAFAH2) (a kind gift of Dr. Hiroyuki Arai, University of Tokyo).

3. Results and discussion

Gene expression was assessed in the whole brain of 10-week-old mice to explore changes in transcription that may precede degenerative changes. Whole brains were collected from three 10-week-old male *Cln1*- and *Cln3*-knockout and three age-matched male wild-type control mice for each

Table 1
Annotated list of genes with altered expression of greater than 1.5 in whole brain of *Cln1*-knockout mice as compared to wild-type controls

Fold change	Accession #	Annotation
5.81	AW259399	Platelet activating factor acetyl hydrolase 2
5.11	AI845935	G-protein β-1 subunit
3.99	AI505203	IAP1
3.9	X02801	GFAP
2.79	AU017193	Unknown
2.33	AW122659	Similar to α-2-adrenergic receptor
2.28	U29055	G-protein β-36 subunit
2.27	AI848373	Unknown
2.08	AV274188	Unknown
1.97	AI837340	VAMP3
1.77	AA606998	Similar to zinc finger protein 97
1.63	AI851048	RAB6
−4.27	AI505387	Unknown
−3.19	U65592	K^+-channel, β-subunit
−2.77	AA874329	Unknown
−2.4	AV150568	Unknown
−2.08	AI849615	GAS5
−1.92	AV848986	Unknown
−1.9	AW125152	Six3
−1.56	AI837101	Calsyntenin-1

Fold change in expression evident in a nine-ways comparison, each wild-type ($n = 3$) compared to each *Cln1*-knockout ($n = 3$). Statistical significance for each change reported has a P-value of < 0.05 by a standard t-test performed in Microarray Suite 5. List derived from genes that had an increase or decrease in expression of 1.5 or more.

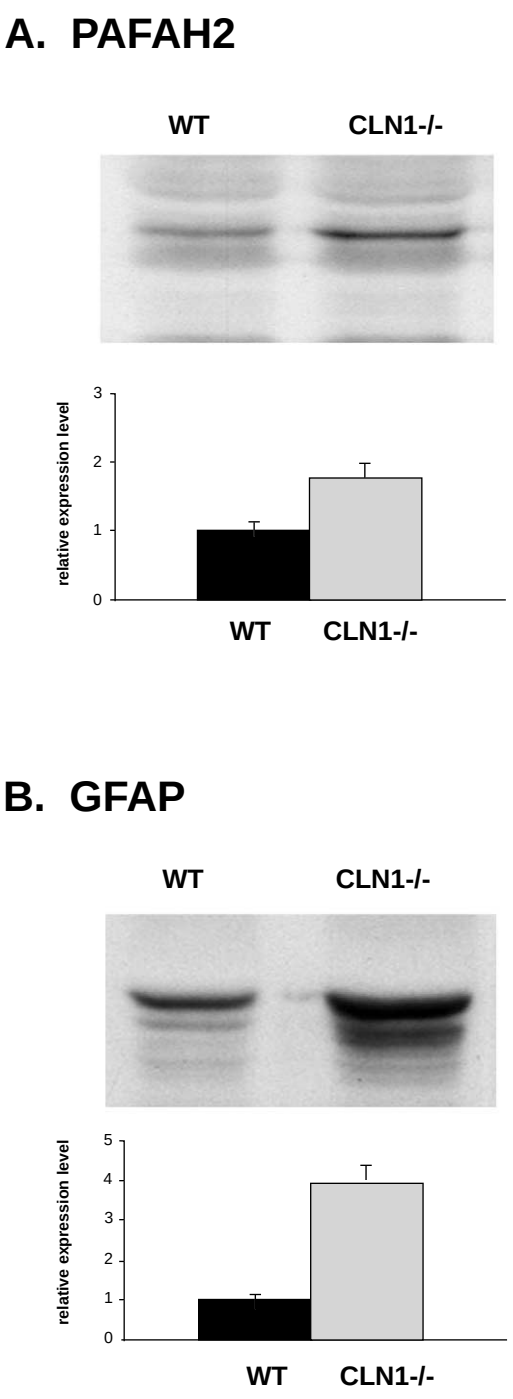

Fig. 1. Immunoblotting and densitometric quantitation shows that (A) PAFAH2 and (B) GFAP are elevated in *Cln1*-knockout mouse brain, as compared to wild-type (WT) mouse brain. 50 μg of protein from *Cln1*-knockout or wild-type brain was analyzed in each of six independent experiments with protein extracted from six brains per experiment. The average signal for wild-type was set to 1, and the error bars indicate the standard error.

knockout model, and the material from each genotype was pooled together for extraction of mRNA. We analyzed age-matched *Cln1*- and *Cln3*-knockout and wild-type control mice. To average out variability among animals and litters, for each genotype RNA was prepared from three pools of three brains each. Expression values for wild-type were compared pairwise with those for knockout, giving nine comparisons for Cln1 and 9 for Cln3. Tables 1 and 2 present the fold changes >1.5 and with $P<0.05$ according to a *t*-test performed in Microarray Suite 5 (Affymetrix). A full data-set reporting all genes, sorted into functional categories, with expression changes of 1–1.5 with a P-*value of* <0.05 *is available at* http://dbb.urmc.rochester.edu/labs/pearce/microarray.html.

3.1. *Altered gene expression in* Cln1-*knockout brain*

Table 1 shows a list of genes that showed altered expression (1.5-fold or more) in 10-week-old *Cln1*-knockout mouse brain as compared to normal. A previous study of *Cln1*-knockout mice [15] showed that the mean time to the development of neurological abnormalities was 21 weeks, and that only 10% of mice demonstrated spasticity at 10 weeks of age, the age at which the current study was performed. It is interesting that relatively few genes showed altered expression, which presumably reflects the pre-symptomatic state. The types of genes with altered expression in the *Cln1*-knockout brain provide potential insights into the molecular mechanisms that are altered. For example, the most highly up-regulated gene in the *Cln1*-knockout brain was PAFAH2, an enzyme of potential interest for two reasons. Firstly, it is a lipase with a rather broad substrate specificity, not unlike PPT1. Therefore, there is a possibility that PAFAH2 is up-regulated in response to the accumulation of lipid substrate, and a role for PAFAH2 in compensating partially for the deficiency of PPT1 can be envisioned. Secondly, PAFAH2 has been shown to translocate to cell membranes in response to oxidative stress, where it is believed to function in the removal of oxidized fatty acids from oxidatively damaged phospholipids [17]. Oxidative stress has been considered a potential contributor to the pathophysiology of lipofuscin accumulation (reviewed in [18]). We have further shown that fatty acyl cysteine thioesters accumulate in the lysosomes of PPT-deficient cells [19]. It is possible that the undigested fatty acids eventually become oxidized upon prolonged storage in the environment of the lysosome, and eventually make their way into PAFAH2 metabolic pathway. The

Table 2
Annotated list of genes with altered expression of greater than 1.5 in whole brain of *Cln3*-knockout mice as compared to wild-type controls

Fold change	Accession #	Annotation
7.32	AI505387	Unknown
5.7	AV148864	Unknown (similar to M5-14)
3.87	AI835219	G-protein γ-4 subunit
2.32	AA681694	Possible RNA binding protein
2.3	X04097	RP2, androgen regulated protein
1.96	AV371646	Unknown
1.92	AW209050	Unknown (similar to human FLJ23151)
1.85	AW122327	Similar to laminin β-2.
−4.43	AA015150	PRK2, lipid activated kinase
−3.56	AF076156	COMT
−3.19	AW046827	Unknown
−2.01	AI848384	Glutamine synthetase
−1.78	AV004774	mGluR3
−1.5	AI843865	Similarity to acetylcholinesterase

Fold change in expression evident in a nine-ways comparison, each wild-type ($n=3$) compared to each *Cln3*-knockout ($n=3$). Statistical significance for each change reported has a *P*-value of <0.05 by a standard *t*-test performed in Microarray Suite 5. List derived from genes that had an increase or decrease in expression of 1.5 or more.

up-regulation of PAFAH2 protein was confirmed by immunoblotting, as shown in Fig. 1, panel A.

It is interesting that mRNAs for both VAMP3 and RAB6 are up-regulated. Both VAMP3/cellubrevin and Rab6 were recently shown to play rather specific roles in trafficking of proteins from early endosome/recycling endosomes to the trans-Golgi network [20]. The significance of this finding remains to be elaborated, but it is likely that dysfunction in one compartment (the lysosome) may have an impact on trafficking in others. Up-regulation of GFAP mRNA is very consistent with the glial cell proliferation that has been well-described in INCL. Immunoblotting for GFAP (Fig. 1, panel B) shows that the protein levels are up-regulated as well. Up-regulation of the G-protein β-1 subunit (same transcript as G-protein β-36 subunit) at the protein level was not confirmed (data not shown). The up-regulation of IAP1 (inhibitor of apoptosis-1) is potentially interesting, because widespread neuronal apoptosis was demonstrated in CLN1 knockout mice [14]. We performed immunoblots to examine IAP1 levels in CLN1 knockout and control mouse brains, but found a great deal of variability in the signal intensity from sample to sample regardless of genotype. IAP1 is regulated in cells by proteolysis and not much is known concerning the role of transcription in its regulation. It is likely that our finding is important but more work will be necessary to better understand the role of IAP1 in INCL and in neurodegenerative disease in general.

Down-regulation of several known genes in CLN1 knockouts was observed (Table 1). One of these is a neural-specific K^+ channel β-subunit. The function of this subunit is to enhance expression of the α-subunit, Kv2.2, a Shab K^+ channel [21]. Other down-regulated transcripts include GAS5, a non-coding growth arrest-specific gene involved in the processing of rRNAs [22], and Six3, a transcription factor important in the development of the eye and forebrain [23]. Also down-regulated was calsyntenin-1, a newly described transmembrane protein with a cytoplasmic calcium binding domain localized to the postsynaptic membrane of both excitatory and inhibitory synapses [24]. Its down-regulation may merely reflect neuronal loss. However, since few neuron-specific genes were identified at this early time, it is more likely that the regulation is specific and warrants further investigation.

It may also be of interest that the most down-regulated gene in CLN1 knockout mice corresponded to an unknown transcript (AI505387) that was the most highly up-regulated gene in CLN3 knockout mice (see below).

3.2. *Altered gene expression in* Cln3-*knockout brain*

Table 2 shows those genes that have increased or decreased expression of 1.5 or more in *Cln3*-knockout mice brain compared to normal. It is interesting that gene products with altered expression in *Cln3*-knockout mice brain that have a known function are associated to neurotransmitter metabolism. Two genes associated with glutamate utilization, namely glutamine synthetase which converts glutamate to glutamine and the glutamate receptor mGluR3 are down-regulated. Down-regulation of genes associated to glutamate utilization has been previously reported, and verified at the protein level in the cerebellum of *Cln3*-knockout mice due to the presence of an autoantibody to GAD65 which inhibits conversion of glutamate to GABA and therefore leads to an accumulation of glutamate [25,26]. This report indicates that the effect of the autoantibody on glutamate levels occurs throughout the brain, and may be a contributory factor to loss of GABAergic neurons in Batten disease [16,25]. Down-regulation of catechol-o-methyltransferase (COMT) is also apparent in the whole brain (Table 2), suggesting that metabolism of dopamine is altered in the *Cln3*-knockout mouse. We have verified that COMT protein levels are in fact decreased in whole brain of *Cln3*-knockout mice as compared to normal, as shown in Fig. 2. The decrease in COMT-protein levels may be linked to a recent report demonstrating a lower density of striatal dopamine D1 receptors in patients with JNCL [27], by way of an underlying disruption in dopamine neurotransmission. Other

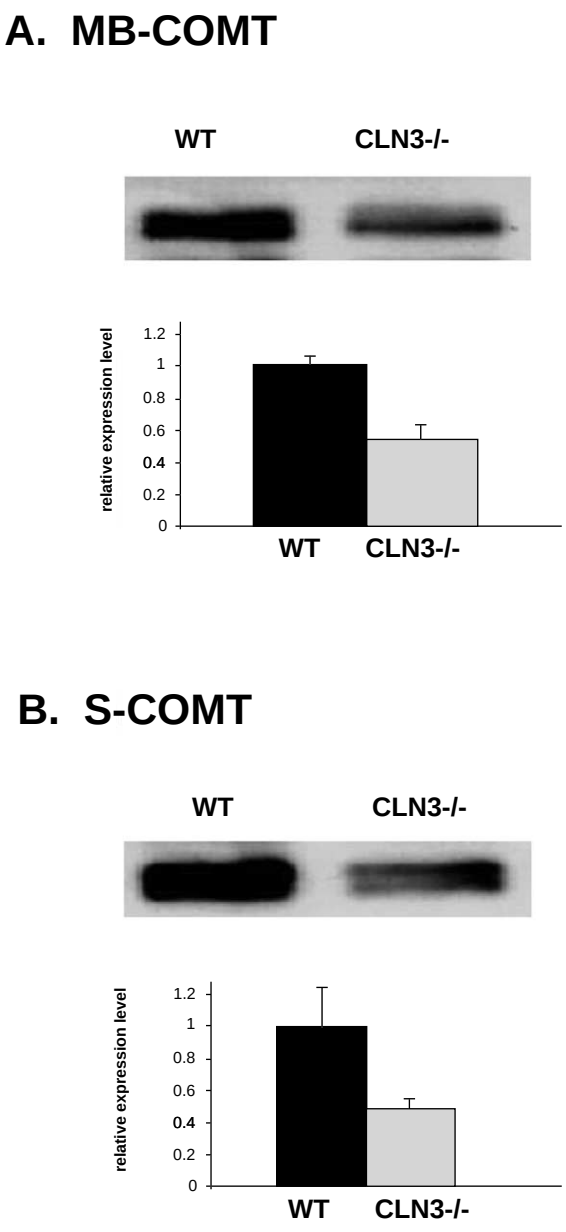

Fig. 2. Immunoblotting and densitometric quantitation shows that COMT is decreased in *Cln3*-knockout as compared to wild-type (WT) mouse brain. A: Membrane bound (MB)-COMT. B: Soluble (S)-COMT in protein extracts from wild-type and *Cln3*-knockout mice. 10 μg of protein from *Cln3*-knockout or wild-type brain was analyzed in each of six independent experiments with protein extracted from six brains per experiment. The average signal for wild-type was set to 1, and the error bars indicate the standard error.

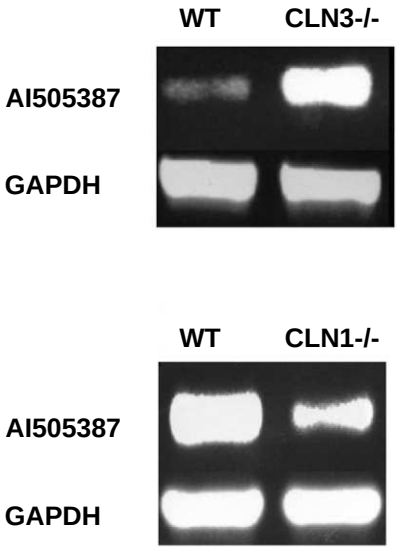

Fig. 3. RT-PCR confirms increased and decreased expression of AI505387 in *Cln3*- and *Cln1*-knockout mouse brain, respectively. RT-PCR of AI505387 as compared to GAPDH in wild-type and *Cln3*- and *Cln1*-knockout mice shows that AI505387 is increased and decreased approximately four-fold in *Cln1*-knockout and *Cln3*-knockout as compared to control wild-type (WT).

imaging studies in JNCL patients reported decreases in fluorodopa uptake [28] as well as in dopamine transporter binding [29], indicating that there may be multiple aspects in which dopaminergic function is perturbed. Disruptions in dopamine neurotransmission have been associated to Parkinson's disease, and implicated in various neuropsychiatric disturbances, components of both of which have similar manifestations in patients during the course of JNCL. In addition, acetylcholinesterase is also down-regulated in *Cln3*-knockout brain suggesting that there may be a disturbance in acetylcholine levels which also could be a contributor to neurological abnormalities in Batten disease.

3.3. *Comparative analysis of gene expression changes between* Cln1- *and* Cln3-*knockout mice*

In comparing the genes that have altered expression in *Cln1*- to *Cln3*-knockout mice brain there are two salient observations. First, there is little overlap between the two NCL-mouse models in the changes seen. Therefore the pre-symptomatic changes at the molecular level are different in *Cln1*- to *Cln3*-knockout mice and is strongly suggestive that despite many similarities, the molecular basis for the pathology of INCL and JNCL is distinct. The second significant observation is that the gene showing the highest degree of down-regulation in the *Cln1*-knockout (unknown gene, accession # AI505387) is the most highly up-regulated transcript in the *Cln3*-knockout. We have validated this observation by semi-quantitative RT-PCR, shown in Fig. 3. The significance of this observation awaits identification of the function of this uniquely regulated gene.

Lysosomal storage diseases such as the NCLs typically result in neurological disease. It would therefore be interesting to compare gene expression changes reported in this study that are present in other mouse models for lysosomal storage disease, such as those reported for Sandhoff disease which highlighted the inflammatory response in this disease [30], and others when they become available. Such an approach would be useful in defining the molecular processes that are disturbed as a result of lysosomal storage in the brain, and also as in this study, in delineating disease-specific alterations at the gene expression level.

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