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Cerebellar defects in a mouse model of juvenile neuronal ceroid lipofuscinosis

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Abstract

Juvenile neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is a neurodegenerative disease resulting from a mutation in *CLN3*, which presents clinically with visual deterioration, seizures, motor impairments, cognitive decline, hallucinations, loss of circadian rhythm, and premature death in the late-twenties to early-thirties. Using a *Cln3* null (*Cln3*^{-/-}) mouse, we report here several deficits in the cerebellum in the absence of *Cln3*, including cell loss and early onset motor deficits. Surprisingly, early onset glial activation and selective neuronal loss within the mature fastigial pathway of the deep cerebellar nuclei (DCN), a region critical for balance and coordination, are seen in many regions of the *Cln3*^{-/-} cerebellum. Additionally, there is a loss of Purkinje cells (PC) in regions of robust Bergmann glia activation in *Cln3*^{-/-} mice and human JNCL post-mortem cerebellum. Moreover, the *Cln3*^{-/-} cerebellum had a mis-regulation in granule cell proliferation and maintenance of PC dendritic arborization and spine density. Overall, this study defines a novel multi-faceted, early-onset cerebellar disruption in the *Cln3* null brain, including glial activation, cell loss, and aberrant cell proliferation and differentiation. These early alterations in the maturation of the cerebellum could underlie some of the motor deficits and pathological changes seen in JNCL patients.

Keywords

Batten disease; *CLN3*; Fastigial; Astroglia; Bergmann glia

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Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.brainres.2009.02.009.

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs), a family of autosomal recessively inherited neurological disorders, are characterized by the accumulation of autofluorescent storage material within cells. The most prevalent form of NCL, juvenile NCL (JNCL), results from gene mutations in *CLN3*. Clinically, JNCL manifests around the age of five with progressive visual deterioration resulting in complete blindness. Patients typically experience seizures, motor deterioration, cognitive impairment, and premature death by the third to fourth decade of life. Clinical studies demonstrate that motor deficits are one of the primary clinical features of JNCL (Raininko et al., 1990). Atrophy within the cerebellum of JNCL patients is observed using magnetic resonance imaging (MRI), which suggests that deterioration within this region contributes to deficits in balance and fine motor coordination in patients (Autti et al., 1996; Nardocci et al., 1995). However, the precise mechanism of cellular dysfunction within the cerebellum has not been characterized.

Previous studies using a *Cln3*^{-/-} mouse model of JNCL demonstrated a marked deficiency in motor coordination and balance by two-months of age (Kovacs et al., 2006). Here, we demonstrate that this decreased motor performance is present by as early as two-week postnatal and persists throughout the shortened lifespan of these mice. Similar to previous findings in adult mice, there is a marked GFAP⁺ and F4/80⁺ glial activation in the *Cln3*^{-/-} cerebellum by one-week postnatal. Additionally, there is a dendritic mis-orientation and increase in dendritic spine density on PCs in the absence of *Cln3*. Close examination of the astrocytic activation revealed small clusters of GFAP⁺ Bergmann glia associated with regions devoid of PCs in the *Cln3*^{-/-} mice. Similar regions of abnormally activated Bergmann glia were observed in human post-mortem JNCL cerebellar tissue. Moreover, there is a selective neuronal loss within the mature fastigial pathway, the medial output circuit of the cerebellum, and alterations in granule cell proliferation within the developing cerebellum. These findings define a novel mechanism of early onset glial activation and cell loss within the developing cerebellum, which could underlie the previously described cerebellar-linked motor dysfunction in JNCL patients.

2. Results

2.1. Motor deficits and glial activation in *Cln3*^{-/-} mice indicative of cerebellar dysfunction

Deficits in motor coordination are one of the primary clinical manifestations of JNCL. Prior studies using a mouse model of JNCL, the *Cln3*^{-/-} mouse, have demonstrated a defect in balance and coordination at two-months of age as measured by accelerating rotorod, a standard test for cerebellar associated motor deficits (Kovacs et al., 2006). To establish the age of onset and progression of these motor deficits, we performed rotorod analysis at different stages of disease progression, including 14, 30, 60, 100 and 180 days old. At all ages measured, *Cln3*^{-/-} mice remained on the rotating rod for significantly shorter amounts of time than wild-type mice, indicating a coordination deficit in these mutant mice (Fig. 1A). Although mice have not reached maturity at fourteen days postnatal, their motor skills were sufficient to enable them to remain on the accelerating rotorod for analysis, revealing a marked deficit in motor skills at this early time point. The deficits in balance and coordination in the *Cln3*^{-/-} mouse do not appear to be progressive over the life-span of the mouse. Although *Cln3*^{-/-} mouse show a similar shortened life-span as JNCL patients (with a sharp decline in probability of survival starting at 13 months and reaching approximately 0.55 by 20 months compared to 0.8 in wild-types mice at the same age; Supp. Fig. 1), a similar age-related progression in motor deficits, in JNCL patients, is not observed.

To confirm that these motor deficits are not the result of decreased muscle tone or metabolic deficits, which could contribute to increased exhaustion and subsequent changes in the

animal's motor coordination, we assayed each of these parameters. Measurements of forearm muscle strength using a wire mesh hang immediately following rotorod testing revealed no difference in muscle tone (data not shown). Furthermore, measurements of metabolic parameters immediately following behavioral assessment showed no significant changes in blood levels of pH, partial CO₂ (pCO₂), lactate, and glucose (pH: wild-type 7.25±0.035, *Cln3*^{-/-} 7.22±0.049; pCO₂ (mm Hg): wild-type 49.94±4.510, *Cln3*^{-/-} 54.18±5.010; lactate (mmol/L): wild-type 7.944±0.703, *Cln3*^{-/-} 8.817±1.450; glucose (mg/dL): wild-type 205.0±15.32, *Cln3*^{-/-} 192.8±18.10). Collectively, these findings demonstrate that loss of *Cln3* results in a cerebellar specific impairment in motor coordination.

To uncover anatomical or cellular changes underlying deficits in motor coordination, we first examined the cytoarchitecture of the *Cln3*^{-/-} mouse. Slight deficits in cerebellar folia structure have previously been shown to have profound effects on motor coordination. Examination of Nissl stained sections of the *Cln3*^{-/-} cerebellum revealed no obvious changes in the gross anatomical structure at three-months of age (Supp. Fig. 2). Although these findings do not preclude the possibility of finer anatomical abnormalities, they suggest that gross anatomical deficits in the cerebellum do not underlie the motor coordination deficits reported here in *Cln3*^{-/-} mouse.

Next, we explored whether an early onset glial activation could contribute to deficits in motor coordination. Previous studies have revealed an increase in the levels of the astrocytic activation marker, glial fibrillary acidic protein (GFAP), and a marker of microglial activation, F4/80, in the mature *Cln3*^{-/-} brains (Pontikis et al., 2004). Similar investigation of glial activation at one-week postnatal showed region specific changes in both GFAP and F4/80 activation (Figs. 1B–C). To account for continued cerebellar development and a lack of maturity in cellular lamination, the lateral hemisphere and vermis of the cerebellum were subdivided into either white matter regions, which includes the external granular cell layer and portions of the cerebellum that will form the molecular layer; or cell layers regions, which include the internal granular cell layer and the Purkinje cell layer. Measurement of the levels of GFAP immunoreactivity revealed elevated astrocytic activation within the white matter of the lateral hemisphere and vermis, as well as the cell layers of the vermis (Fig. 1B). Furthermore, microglial activation, measured by F4/80 immunoreactivity, was seen in the white matter of the lateral hemisphere and cell layers of the vermis in the *Cln3*^{-/-} cerebellum (Fig. 1C). These findings reveal an early onset glial activation in the cerebellum of *Cln3*^{-/-} mice that appears to parallel deficits in motor coordination.

2.2. Regions of Purkinje cell loss and Bergmann glia activation

At one-week postnatal, the most prominent astrocytic cell population within the developing cerebellum is the Bergmann glia. These cells serve as the migratory guides for transport of immature granule cells from the external to internal portions of the cerebellum. Close examination of the GFAP⁺ astrocytes within the *Cln3*^{-/-} cerebellum revealed that these activated cells displayed the characteristic morphology of Bergmann glia, with a soma in the Purkinje cell layer and extended reactive processes across the molecular layer, terminating near the pial surface of the cerebellum (Figs. 2B–B', C–C'). Furthermore, these regions of activation were seen in discrete pockets or clusters across the cerebellar folia.

Activation of Bergmann glia can result from morphological changes within neighboring Purkinje cells. In sections immunolabeled with antibodies to anti-GFAP and anti-calbindin, a calcium binding protein expressed in Purkinje cells, it was observed that each cluster of activated Bergmann glia resided in a region devoid of Purkinje cells (Figs. 2B', C'; asterisks). The cell bodies of the Purkinje cells neighboring these clusters appeared normal but their processes were severely misshapen, losing ordered orientation as they ascend towards the pial surface (Fig. 2G). These findings demonstrate the presence of discrete

clusters of activated Bergmann glia and suggest a close association between these regions of astrogliosis and disorganization and/or loss of Purkinje cells in the *Cln3*^{-/-} cerebellum.

To establish that these clusters of activated Bergmann glia are indeed associated with Purkinje cell loss (and not simply down-regulation of calbindin), we immunolabeled sections with antibodies to anti-GFAP and anti-MAP2, a microtubule associated protein expressed in neuronal dendrites and cell soma. Again, in regions of GFAP⁺ Bergmann glia, there was a complete absence of MAP2⁺ Purkinje cell bodies in *Cln3*^{-/-} cerebellum (Fig. 2E), confirming that these regions of Bergmann glial cell activation occur in conjunction with the loss of Purkinje cells. Furthermore, MAP2 staining also revealed the disorganization in the cellular lamination at the granular/Purkinje cell layer junction with the Purkinje cell layer often infiltrated by a smaller diameter cells, possibly displaced granule cells or GABA-positive interneurons resident to the granule cell layer (Figs. 2E, G arrowheads). Anti-MAP2 immunolabeling further confirmed an apparent disruption in the normal pial directed orientation of Purkinje cell processes within the molecular layer (Fig. 2E) compared to wild-type. Moreover, this GFAP⁺, Bergmann glia activation was most prominent in the vermis with numerous clusters located within each folia (data not shown). Although, GFAP⁺ activation was seen in the lateral hemisphere, immunolabeling was much less frequent with occasional activated cell found within the white matter of every third or fourth folia examined (data not shown). Combined, these findings demonstrate profound Bergmann glial activation with central regions of the developing *Cln3*^{-/-} cerebellum.

2.3. Glial activation and loss of Purkinje cells in JNCL patients

To explore whether similar Bergmann glial specific activation and loss of Purkinje cells occurs in diseased JNCL patients, we immunolabeled human post-mortem cerebellar samples with anti-GFAP and anti-calbindin antibodies. In JNCL autopsy material there was increased GFAP⁺ processes throughout the molecular layer (Figs. 3B–D). Similar to the *Cln3*^{-/-} mice, these regions of intense GFAP⁺ immunoreactivity were located in regions devoid of calbindin⁺ Purkinje cells (Figs. 3B–C). Control cerebellar tissue showed limited GFAP⁺ staining and robust calbindin⁺ Purkinje cells (Fig. 3A). Comparing JNCL cases with varying degrees of progression, the degree of Purkinje cell loss and the intensity of GFAP immunoreactivity within Bergman glia was less pronounced in a more slowly progressing (death at 22 years, Fig. 3B) vs. more rapidly progressing JNCL case (death at 16 years, Fig. 3C), which more closely resembled the phenotype of late infantile NCL (LINCL) autopsy material (Fig. 3D) as previously reported (Chang et al., 2008). These findings demonstrate that the pathological changes we report here in the cerebellum from *Cln3*^{-/-} mice are similar to those present in afflicted JNCL patients. Thus, this unique Bergmann glial activation and loss of Purkinje cells could, in part, underlie the motor deficits reported NCL patients.

2.4. Purkinje cell morphology

As anti-calbindin and anti-MAP2 immunolabeling suggested structural alterations in Purkinje cells adjacent to activated glia (Fig. 2), we closely examined the morphology of Purkinje cell in the *Cln3*^{-/-} cerebellum. Purkinje cells immunolabeled with anti-calbindin antibodies revealed a number of cells that display the atypical dendritic orientation in *Cln3*^{-/-} mice, with the main proximal dendrite pointing obliquely across the molecular layer (Fig. 4A, arrow). Alterations in dendritic morphology, including meganeurites and ectopic dendritogenesis, are hallmarks of many lysosomal storage disorders (Bjurulf et al., 2008; van der Voorn et al., 2004; Walkley et al., 1990, 2005). Therefore, we explored whether similar defects existed in the dendrites of *Cln3*^{-/-} Purkinje cells. Surprisingly, there was no apparent meganeurites in the cerebellum of these mice. Interestingly though, measurements of the angle of the apical dendrite to the pial surface in Golgi impregnated sections revealed a significant decrease in the angle in the *Cln3*^{-/-} mice (Fig. 4B). Similar to previous

observation with anti-MAP2 immunolabeling (Fig. 2), the dendritic tree of these cells seemed less elaborate in the Golgi impregnated *Cln3*^{-/-} cerebellum. High resolution imaging allowed quantification of the number of Purkinje cell dendritic spines in the *Cln3*^{-/-} cerebellum. Surprisingly, there was a selective increase in the number of spines in the *Cln3*^{-/-} mice, specifically in the left side of the vermis with the remaining three regions unchanged (Fig. 4C). This suggests a spatially restricted disruption of Purkinje cell dendritogenesis. These findings further indicate a specific insult in *Cln3*^{-/-} mice that severely affects the cellular integrity of neurons within central pathways of the cerebellum.

2.5. Cell loss in the medial deep cerebellar nuclei

To further explore the consequence of Purkinje cell loss and glial activation in the *Cln3*^{-/-} cerebellum, we examined whether there were changes in neuronal cell populations receiving direct inputs from Purkinje cells, specifically the single efferent output of the cerebellum to DCN. The medial DCN receives projections from the Purkinje cells of the vermis and the intermediate and lateral DCN receiving projections from the more lateral portion of the cerebellum. Therefore, to determine whether a specific cerebellar output pathway was disrupted in *Cln3*^{-/-} mice we performed morphometric analysis of the DCN (Figs. 5A–B). First, Cavalieri estimates of the volume of each DCN were obtained to determine whether there was any overall atrophy in these nuclei. This analysis revealed no significant shrinkage in the overall volume of any of the *Cln3*^{-/-} DCN at six-months of age (Fig. 5C). Second, to investigate whether there were changes in the number of DCN cells, unbiased fractionator cell counts were taken within each DCN. Nissl stained cells in the medial, lateral, and intermediate nuclei were classified based on their size as either large neurons, consisting of the phenotypic DCN projection neurons, or as small cells, including inhibitory interneurons, glia, and endothelial cells. Cells counts revealed a significant reduction in the number of large projection neurons within the medial nuclei of the DCN, constituting the fastigial output pathway, while the intermediate and lateral DCN were unaffected (Fig. 5E). This reduction corresponds to a loss of nearly half of the large projection neurons in the *Cln3*^{-/-} medial DCN compared to wild-type mice (1068 cells vs. 2189 cells). In contrast there were no changes in the number of small cell populations, which is comprised of interneurons, glia, and endothelial cells, in any of the DCN (Fig. 5F). To discern whether this neuronal loss affected the overall size of remaining neurons in the DCN, unbiased nucleator estimates of cell volume were taken and showed no significant changes in the area of remaining cells in the medial DCN, nor in the intermediate or lateral DCN (Fig. 5D). Thus, these findings combined demonstrate a profound insult to the fastigial output pathway of the *Cln3*^{-/-} cerebellum, including prominent Bergmann glial activation as well as Purkinje cell loss within the vermis and neuronal loss in the medial DCN.

2.6. Alteration in cellular proliferation

Thus far, we have described alterations in both the Purkinje cell integrity and gliosis within medial portions of the cerebellum. These affected Purkinje cells and Bergmann glia provide trophic and structural support of neighboring cells within the developing cerebellum. For instance, cerebellar granule neurons (CGNs) are actively proliferating, migrating, and differentiating during the first few weeks of postnatal life in the mouse and rely heavily on interactions with Purkinje cells and Bergmann glia for their placement and survival. Therefore, we characterized the initial stages of cerebellar granule cells development in the *Cln3*^{-/-} mice. Normally, granule cells undergo proliferation in the external granule cell layer, differentiate, and migrate radially to their final residence in the internal granule cell layer. Thinning of the internal granule cell layer, for instance, due to deficiencies in adequate cell migration, may contribute to the cerebellar-specific motor abnormalities described here. Indeed, measurements of granule cell layer thickness at one-week postnatal

revealed a significantly reduced thickness of the early postnatal internal granule cell layers in *Cln3*^{-/-} mice (Fig. 6A).

In order to establish whether this thinning of the granule cell layer is a consequence of altered proliferation in the external proliferative zones, cerebella from one-week postnatal *Cln3*^{-/-} and wild-type mice were dissociated and fractionated into two cell populations, an upper fraction including astrocytes and Purkinje cells and a lower fraction including proliferating CGNs. Surprisingly, *Cln3*^{-/-} CGNs showed a significantly higher percentage of BrdU incorporation, thymidine analog that marks the S-phase of the cell cycle, indicating of increase in cell proliferation (Fig. 6B). Furthermore, the upper fraction of cells, consisting of astrocytes, microglia, endothelial cells, and glial progenitor cells, showed a elevation in the percentage of BrdU⁺ cells in the *Cln3*^{-/-} compared to wild-type (Fig. 6C), demonstrating a global disruption in cell proliferation.

Little is known about the precise function of CLN3, particularly during nervous system development. One possibility is that CLN3 is directly influencing signaling pathways critical for the proliferation, migration and differentiation of neurons in the developing cerebellum. One candidate we examined is thyroid hormone (TH) which plays a crucial role in cerebellar development and reported alterations in this signaling pathway show some commonality to those observed in the *Cln3*^{-/-} cerebellum. Thus, interplay between CLN3 and TH signaling could result in many of the cellular and behavioral changes observed in the absence of CLN3. To investigate whether changes in TH levels indeed contribute, we performed Western blot analysis on cerebella using an anti-TH antibody at various time-points (P7, P14, and adult) and observed no changes in the levels of this hormone (data not shown).

Alternatively, alterations in Notch signaling, critical for the regulation of cell division within the external granule cell layer, could influence *Cln3* regulation. Notch activation inhibits the differentiation of granule cell progenitors, maintaining them in a proliferative state, and ultimately influences the fate of this cell population. Recently, studies in *Drosophila* have demonstrated a genetic interaction between *CLN3* and Notch (Tuxworth et al., 2009). Therefore, we investigated whether factors involved in the Notch signaling pathway including the Notch receptors 1 and 2; the Notch ligand, Jagged1; a downstream target of Notch signaling, Hes1; a CGN specific mitogen, Sonic hedgehog (Shh); and a proneural transcription factor regulated by Hes1 expression, Math1 were changed in the *Cln3*^{-/-} cerebellum. Quantitative reverse transcriptase PCR (qRT-PCR) revealed a significant elevation in the levels of Notch2 at P7 in the *Cln3*^{-/-} cerebellum (Fig. 6E). A temporal examination of these factors during the first week of postnatal cerebellar development showed that Notch2 expression in *Cln3*^{-/-} mice was increased at least 10-fold over wild-type samples at each time point examined (Figs. 6D, E). Furthermore, Notch2 expression remains elevated after the typical peak in expression, which occurs around P7 and diminishes around P14 (data not shown). In identical samples, the expression of Notch1 and Hes1 (Figs. 6D, E) was unchanged, suggesting that these alterations in proliferation are specific to Notch2 regulated mechanisms. Expression of Math1 did decrease significantly at P7, suggesting that increased Notch2 is preventing the expression of this proneural gene (Fig. 6D). Alterations in these components of the Notch signaling pathway could contribute to or be the direct result of the altered neuronal proliferation apparent in the *Cln3*^{-/-} cerebellum. Collectively, this study demonstrates that although granule cells are capable of actively proliferating in the exterior confines of the developing cerebellum, they are unable to properly migrate or fully mature/survive once they reach their final destination, possibly owing to a deficiency in the structural and trophic support provided by the Purkinje cells and Bergmann glia.

3. Discussion

In this study, we demonstrate a novel early onset motor deterioration accompanied by a robust activation of astrocytes and microglia in the developing cerebellum in the absence of *Cln3*. Furthermore, in the *Cln3*^{-/-} cerebellum, there is targeted disruption in the medial, fastigial pathway, including activation of Bergmann glia, loss of Purkinje cells and loss of neurons in the medial deep cerebellar nuclei. Magnetic resonance imaging (MRI) studies of motor system deterioration have shown early changes within the cerebellum of diseased patients (Autti et al., 1996; Nardocci et al., 1995). Similarly, studies using computer tomography (CT) scanning techniques, have shown atrophy within the cerebellum in the vermis and lateral hemispheres of JNCL patients, detectable around the age of nine years (Raininko et al., 1990). Comparison of clinical data collected from the patients with imaging data showed a close correlation in atrophy of cerebellar hemispheres and decrease in motor function, balance, and coordination (Raininko et al., 1990). These studies combined suggest that human JNCL patients are succumbing to early anatomical deficits within the cerebellum resulting in progressive motor decline similar to those reported here in the mouse model of JNCL.

3.1. Motor coordination and cerebellum defects in *Cln3*^{-/-}

Previous studies have demonstrated rotorod specific motor deficits in the *Cln3*^{-/-} mice (Kovacs et al., 2006). Here, we expand on these earlier studies, demonstrating that this motor decline occurs much earlier than previously reported. Deficits in motor function are seen as early as P14, a time-point when the murine cerebellum is still developing, and persist throughout the lifespan of the mouse. But, are these deficits in motor skills seen in the disease patients and the mouse model entirely a result of cerebellar deterioration? JNCL patients also have changes in extrapyramidal functions that appear to result from cell loss in the basal ganglia, which occurs much later than the cerebellar atrophy (Autti et al., 1996; Ruottinen et al., 1997). Similarly, we have observed degenerative changes within the basal ganglia of the *Cln3*^{-/-} mice (Weimer et al., 2007). The early onset age of cerebellar associated motor defects reported here is unique. Furthermore, in *Cln3*^{-/-} mice, this cerebellar specific motor deficit does not appear to regress over time, suggesting that additional extrapyramidal deterioration could confound these insults of the motor systems, and this explains why decline in motor function progresses over the course of Batten disease.

3.2. Is *Cln3* critical for cerebellar development?

During cerebellar development, Bergmann glia serve as the radial scaffold along which newly generated granule cells migrate from the external to internal regions of the cerebellum (Hartmann et al., 1998; Hatten, 1990; Rakic, 1971). Unlike their radial glial cousins of the developing cerebral cortex, the Bergmann glial scaffold is maintained in the mature brain, providing structure and molecular support to neighboring cells. Although their precise function in the mature brain is unclear, it has been hypothesized that Bergmann glia extend complex side branches that interact with synapses between parallel fibers and the spines of Purkinje dendrites, serving a homeostatic role in such things as uptake of excess glutamate and K⁺ clearance (Blackstone et al., 1992; Muller and Kettenmann, 1995; Muller et al., 1992; Reichenbach et al., 1995). Each of these Bergmann glia cells extend processes that ensheath between 2000 and 6000 Purkinje cell synapses (Reichenbach et al., 1995). Experiments using selective ablation of Bergmann glia led to granule neuron degeneration, alteration in Purkinje cell dendritic arbors, and disruption in the junction between the Purkinje and granule cell layer. Moreover, ablation of this astrocytic population led to severe motor discoordination (Cui et al., 2001). These results mirror those reported here in the *Cln3*^{-/-} mice, with alterations in the dendritic arbors of Purkinje cells, disruption in the cells

located near the junction of the Purkinje and granule cell layers, and motor coordination deficits. Thus, Bergmann glial activation in *Cln3*^{-/-} mice could impair the function of these cells, leading to changes similar to those reported following complete cell ablation.

Although it is clear that these combinatorial insults to the *Cln3*^{-/-} cerebellum result in profound motor deficits, it is not clear which comes first — the glial activation, alterations in dendritic arborization, or selective, topographic neuronal loss. The Bergmann glial activation could contribute to the selective loss of Purkinje cells or, alternatively, this glial activation could be in response to Purkinje cell dysfunction. Therefore, a careful developmental examination of Purkinje cell loss and Bergmann glia activation could provide valuable insight into which event occurs first. Furthermore, additional studies of this mouse model could lend further support to demonstrate that JNCL may, in part, be a disease of the developing brain and not just the result of neurodegeneration, as previously thought. For example, in addition to supplying their own trophic support in the form of brain derived neurotrophic factor (BDNF), Purkinje cells rely on neighboring granule cells for a majority of their BDNF. This trophic support in turn aids in the maintenance of the extensive dendritic arbors of the Purkinje cells both in the developing and mature cerebellum (Adcock et al., 2004; Richardson and Leitch, 2005). Therefore, it is possible that disruption in the granule cells signaling precedes Bergmann glia activation or Purkinje cell loss, leading to diminished trophic support and preventing proper dendritic elaboration in *Cln3*^{-/-} mice. Misoriented dendritic arborization similar to that reported here in the *Cln3*^{-/-} cerebellum has been described in other mouse models and areas of the brain as well, including in the hippocampus, the motor cortex, and in Purkinje cells (Custer et al., 2006; Demyanenko et al., 1999; Grove et al., 2004). Thus, a careful comparison of the temporal and spatial loss of cells within the *Cln3*^{-/-} cerebellum, as well as these other mouse models, could provide valuable insight into specific signaling pathways affected by the loss of the *Cln3*.

One such signaling pathway, which appears to mirror many of the cellular and phenotypic changes, reported here, is the thyroid hormone (TH) signaling pathway. TH levels and signaling through this pathway are critical for postnatal cerebellar development. Alteration in TH signaling machinery lead to aberrant Purkinje cell dendrite arborization, granule cell proliferation/migration, and motor behavioral (Anderson, 2008; Koibuchi, 2008; Pasquini and Adamo, 1994). In addition to alterations in levels of TH, perturbations in the level and/or activity of cerebellar TH interacting and regulatory machinery including the thyroid receptors (TRβ1, TRα2, Bradley et al., 1992); interacting/co-regulated receptors (RXR and RORα; Glass and Rosenfeld, 2000; Koibuchi and Chin, 1998; Koibuchi et al., 2001); corepressors and coactivators (Sin3, p300/CBP, c/CAF, SRC-1, hairless, Misiti et al., 1999; Nishihara et al., 2003; Thompson and Bottcher, 1997; Yousefi et al., 2005), and TH-responsive genes (including neurotrophin 3, Li et al., 2004) have been shown to impart severe alterations on cerebellar development. Although we observed no obvious changes in the levels of TH in the developing or adult cerebellum, a more extensive examination of this signaling pathway could uncover a yet unknown link between thyroid receptor signaling and CLN3.

Two other critical signaling pathways that may be affected by the loss of *Cln3* in the developing cerebellum are the Notch and JNK signaling pathway. Recently, gain-of-function studies in *Drosophila* have shown that ectopic expression of *Cln3* inhibits the Notch signaling pathway (Tuxworth et al., 2009). Furthermore, this altered expression of *Cln3* in *Drosophila* leads to robust activation of the JNK signaling pathway. In our study, we further demonstrate a mechanistic link between *Cln3* and the Notch signaling pathway. Loss *Cln3* in the developing cerebellum leads to robust increases in the levels of Notch2, a member of the Notch signaling pathway shown to be critical for the maintenance of neuronal cell polarity and cytoskeletal rearrangement in the developing brain (Klezovitch et al., 2004;

Roegiers and Jan, 2004), presumably through interactions with known polarity proteins such as members of the Par polarity complex, Lgl, Numb, and Numbl (Ivanov, 2008). Thus, these finding could further support a yet to be explored mechanistic link between *Cln3* and regulation of cell polarity and proliferation in the developing brain.

3.3. Loss of *Cln3* provides a model for studying Purkinje cell integrity and topographic deficits in the cerebellum

The cerebellum receives a wide variety of sensory input from the cerebral cortex, brain stem, and spinal cord to generate motor-related outputs. Purkinje cells receive excitatory input from climbing fibers and parallel fibers to form the single output system of the cerebellum. The axons of the Purkinje cells then extend out of the cerebellum via the deep cerebellar nucleus. Output from the cerebellum, in the form of efferent Purkinje cell projections, is relayed through three deep nuclei: the fastigial (medial), interposed (intermediate), and dentate (lateral) nuclei. In this study, we demonstrate a topographic insult specific to the fastigial nucleus, a region primarily involved with regulating balance by relaying information to the vestibular and reticular nuclei. Whereas the dentate and interposed nuclei, which are involved with voluntary movement by sending axons primarily to the thalamus and the red nucleus, appear unaffected. Studies of several cerebellar-specific mouse mutants have provided useful clues about the mechanisms regulating development/degeneration of these critical cerebellar pathways. Many of these mutant models show a selective loss of subclasses of cells within the cerebellum, including a selective loss of both Purkinje and granule cells in the ROR α mutant-staggerer mutant (Doulazmi et al., 2006; Vogel et al., 2000), the *lurcher* (McFarland et al., 2007), *scrambler* (Goldowitz et al., 1997; Yang et al., 2002), and *reeler* (Badea et al., 2007) mutants. Other cerebellar mutant mice, such as the *stargazer* mutants, show only molecular changes, with minimal cell loss (Payne et al., 2007). Mechanistically, these models have provided insight into deficits in cell migration, proliferation, and atrophy due to loss of tropic support. Combined, these mutant mice provide useful models for our understanding of the development and/or degeneration of critical motor signaling pathways. The *Cln3* mutant mouse employed here provides an additional disease model for understanding how selective cerebellar cell loss, specifically within the fastigial (or medial) pathway of the cerebellum with a near 50% reduction in the number of large neurons in the medial deep cerebellar nuclei (DCN) and selective loss of Purkinje cells, can underlie deficits in the maturation and maintenance of cerebellar specific motor processing.

In addition to this topographic neuronal loss in the medial DCN, we demonstrate changes in the Purkinje cells that project to this region. Surveying the lateral hemispheres and vermis, we have revealed changes in the cytoarchitecture of Purkinje cells within the vermis, with a selective increase in the Purkinje cell dendritic spine number in *Cln3*^{-/-} mice. This increase in spine number may correlate to over activity of these cells, which could alter inhibitory output from the Purkinje cells and correlate with the decreased motor activity observed in these mice. Furthermore, as regions of robust glial activation appear to be devoid of Purkinje cells, this increase in spine number could be reflective of an attempt of the remaining Purkinje cells to maintain cerebellar function by increasing the dendritic arborization.

3.4. Summary

This study provides novel evidence for changes within the cerebellum of *Cln3*^{-/-} mice that reflects neurodegenerative pathogenesis and may suggest a possible developmental component in the pathogenesis of JNCL. We have used the *Cln3*^{-/-} mouse to examine cerebellum integrity and function in the absence of *Cln3*. These *in vivo* findings demonstrate a novel involvement of cerebellar dysfunction, further extending our understanding of the pathogenesis of JNCL beyond the forebrain, which was long thought to be the primary site

of disease. Curiously, it was recently speculated that the mouse model used in this study might retain a portion of *Cln3* (Kitzmüller et al., 2007). However, there was no data presented in support of a transcribed message or protein corresponding to any portion of *CLN3* being present in *Cln3*^{-/-} mice. In a separate study, we have excluded this possibility (Chan et al., 2008) and as such this mouse model is indeed a knockout that reproduces the effect of lacking *CLN3*. Indeed, as this and previous reports emphasize, these mice recapitulate the human disease and remain valid for understanding the pathogenesis of juvenile Batten disease.

In summary, this study describes evidence for focal changes within the cerebellum of *Cln3*^{-/-} mice, including loss of cells within the medial DCN, the primary output nuclei of Purkinje cells in the vermis, as well as severe impairment in motor coordination. We demonstrate activation of discrete clusters of Bergmann glia, loss of proximal Purkinje cells proximal to this characteristic pattern of gliosis, and an increase of Purkinje cell dendritic spines, all of which are restricted to the cerebellar vermis. These morphological changes appear in conjunction with alterations in Purkinje cell dendrites and disruption in cells at the granule and Purkinje cell layer interface. Similar pathological findings in human JNCL patients provides further evidence that molecular and cellular alterations in the integrity of Purkinje cells within the cerebellum contributes, at least in part, to the motor deficits associated with the progression of Batten disease. Continuing work into understanding the function of *CLN3* in both the normal and the pathological condition will hopefully offer insight into the interventions that can be made available to families afflicted with JNCL.

4. Experimental procedures

4.1. Behavioral assessment of *Cln3*^{-/-} mice

4.1.1. Animal husbandry and survival—129/SvJ wild-type and homozygous *Cln3* knockout mice (*Cln3*^{-/-}) on the same background were housed under identical conditions (Mitchison et al., 1999). All procedures were carried out in accordance with NIH guidelines and the University of Rochester Animal Care and Use Committee Guidelines. The survival rate of *Cln3*^{-/-} and wild-type mice was tracked over time.

4.1.2. Rotorod testing—An accelerating rotorod (AccuScan Instruments, Inc., Columbus, OH) was used to measure motor coordination over time in *Cln3*^{-/-} and control mice as previously described (Kovacs et al., 2006). Naive *Cln3*^{-/-} and wild-type mice (*N* of 7–10) at indicated ages were tested. In brief, mice were placed on the rotorod starting at zero rpm and accelerating to 30 rpm over a period of 240 s for a training period to acclimate mice to the rotorod task. Animals were trained for three trials consisting of three consecutive runs. Trials were separated by a thirty minute rest interval. Following training, animals rested for four hours and then were tested for three test trials consisting of three consecutive runs with thirty minutes of rest between each test trial. The three consecutive runs for each test trials were then averaged, the latency to fall during the testing period was calculated and data was analyzed by two-way ANOVA with Bonferroni's post-hoc *t*-test).

4.1.3. Wire mesh forearm strength test—Muscle strength was tested by hanging the mouse by its two front paws on a three mm wire mesh, inverted, and placed twelve inches above a surface. The time until release from the wire mesh was recorded for three consecutive trials.

4.1.4. Blood gas testing—Blood gas testing was performed on six month old *Cln3*^{-/-} and control mice immediately following rotorod testing. Animals were euthanized and arterial blood samples were immediately collected into Pro-Vent Plus arterial blood syringes

containing dry lithium heparin (Portex, Inc., Keene, NH). Measurements of partial CO₂, pH, lactate, and glucose levels were performed in the University of Rochester Blood Bank.

4.2. Histological and stereological characterization of *Cln3*^{-/-} cerebellum

4.2.1. Histological processing and Nissl staining of cerebellum—For histological analysis, brains from six month old homozygous *Cln3*^{-/-} mice and age- and sex-matched wild-type controls (*N*=3) were harvested as previously described (Pontikis et al., 2004). Every second section of serial 40 μm frozen coronal sections through the CNS was stained with the Nissl dye cresyl violet as previously described (Pontikis et al., 2004) to provide direct visualization of neuronal morphology for cellular quantification.

4.2.2. Measurements of regional volume, laminar thickness, neuronal number, and neuronal volume—Unbiased Cavalieri estimates of the volume of the deep cerebellar nuclei were made from each animal, as described previously (Bible et al., 2004; Pontikis et al., 2004), with no prior knowledge of genotype. Regional volumes were expressed in μm³ and the mean volume of each region was calculated for each genotype. *StereoInvestigator* software (MicroBrightField, Inc., Williston, VT) was also used to make measurements of the thickness of the external granule cell layer, molecular layer and internal granule cell layer by drawing a series of lines across each layer perpendicular to the pial surface, as described previously within the cerebral cortex (Bible et al., 2004). These measurements were made in three consecutive sections with ten measurements made for each lamina within each section. *StereoInvestigator* software was used to obtain unbiased optical fractionator estimates of total neuronal numbers in deep cerebellar nuclei and nucleator estimates of neuronal volume, as previously described (Bible et al., 2004). The boundaries of nuclei were defined by reference to landmarks in a mouse brain atlas (Paxinos and Franklin, 2001). 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 (Gundersen and Jensen, 1987). Data were analyzed by two-way ANOVA with Bonferroni's post-hoc *t*-test.

4.2.3. Immunohistochemistry—For immunological examination of *Cln3*^{-/-} and control mouse cerebellum, animals were sacrificed and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed, immersed and fixed in 4% PFA, followed by changes in a sucrose gradient. For immunolabeling of human sections, mouse sections, and cells, samples were blocked in TBS-T plus serum (0.02 M Tris, pH 7.5, 0.01 M NaCl, 5% Normal Goat Serum, 1% Triton X-100) or TBS-T plus 5% dry powdered milk and then incubated in primary antibodies. The following antibodies were used for immunohistochemistry, immunoprecipitations, and immunoblotting experiments: anti-GFAP and anti-calbindin (Sigma, St Louis, MO); anti-MAP2 (Abcam Inc., Cambridge, MA); anti-calbindin (Swant, Bellinzona, Switzerland); anti-GFAP (DAKO, Cambridge, UK). For immunolabeling of tissue and cells, samples were subsequently incubated in appropriate Alexa secondary antibodies (Molecular Probes, Carlsbad, CA). Dendritic spines were counted per micron on a dendritic process in a single plane. Images were collected using a 100× objective on a confocal microscope and were analyzed by counting the number of spines per micron (*N*=3 mice/genotype; five fields per section; five sections per region).

4.2.4. Quantification of GFAP and F4/80 immunoreactivity—To investigate the extent of GFAP and F4/80 immunoreactivity within the postnatal day 7 cerebellum, *Cln3*^{-/-} and wild-type mice (*N*=3) were processed using a modified version of a previously published immunoperoxidase protocol (Bible et al., 2004). Samples were labeled with anti-GFAP and anti-F4/80 antibodies (insert companies) as primary antibody; biotinylated secondary antibody (Vector Laboratories, Peterborough, UK); followed by ABC reagent

(Vectastain Elite ABC kit, Vector Laboratories). Immunoreactivity was visualized by incubation in 0.05% diaminobenzidine tetrahydrochloride (DAB) (Sigma, Poole, UK) and 0.001% H₂O₂ for 10 min. The relative levels of anti-GFAP and anti-F4/80 immunolabeling within the white matter and vermis of the cerebellum was quantified via semiautomated quantitative thresholding image analysis, on sections processed at the same time and stained under identical conditions, as previously described (Bible et al., 2004; Lim et al., 2006; Pontikis et al., 2004); using *Image-Pro Plus* image analysis system (MediaCybernetics, Silver Spring, MD). Briefly, 100 non-overlapping digital RGB images from each mouse were captured via live video camera (JVC, 3CCD, KY-F55B) mounted onto a Zeiss Axioplan microscope (Carl Zeiss (UK) Ltd., Welwyn Garden City, UK) using a 40× objective from equivalent brain regions in a 1 in 12 series. All parameters were held constant during imaging. Data was collected, analyzed, and plotted as the mean percentage area of immunoreactivity±SEM for each phenotype and sub-region of the cerebellum.

4.2.5. Golgi staining of WT and *Cln3*^{-/-} cerebellum—Six month old wild-type and *Cln3*^{-/-} mouse cerebellum (*N*=3) were immersed in Golgi-Cox solution (1% potassium dichromate, 1% mercuric chloride, 0.8% potassium dichromate) for two weeks and then transferred to 30% sucrose for one week. Cerebellum sections (100 μm) were reacted in 50% ammonia solution, washed in PB, incubated in 1% sodium thiosulphate, and washed in PB. Sections were mounted on slides, dehydrated, and impregnated cells were analyzed. The angle of the apical dendrite was measured using Image J and an average of 60 cells per animal measured. Data were analyzed by Student's *t*-test.

4.2.6. Immunohistochemistry of human tissue—CNS tissue from two JNCL patients (age at death 16 years and 22 years of age) and one age matched neurologically normal control (age at death 25 years) were obtained from Human Brain and Spinal Fluid Resource Center, LA, USA. For comparison, one late infantile NCL case (age at death 6 years) was also obtained from the same source. At autopsy, tissues were fixed immediately by immersion in 4% formaldehyde, processed and embedded in paraffin wax. Study protocols for the use of human material were approved by the Ethical Research Committees of the Institute of Psychiatry, UK (approval numbers 223/00, 181/02). 8 μm sections were cut from paraffin embedded blocks of the cerebellar vermis using a sledge microtome (Leica SM2400, Leica Microsystems (UK) Ltd) and processed for immunohistochemistry, as outlined above. Immunofluorescence was observed by conventional epifluorescence microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss Ltd, Welwyn Garden City, UK).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Decreased viability and motor coordination in *Cln3*^{-/-} mice. (A) Rotorod testing was performed on postnatal (P) day 14, 30, 60, 100, and 180 old control wild-type and *Cln3*^{-/-} mice. Data is plotted as latency to fall from the rotorod. *Cln3*^{-/-} mice had a significant reduction in their ability to remain on the rod as it accelerated, at all time points. (B–E) Quantitative analysis of glial activation in wild-type (+/+) and *Cln3*^{-/-} cerebellum. Sections were immunolabeled with either anti-GFAP (B,C) or anti-F4/80 (D, E) antibodies and the percent immunoreactivity was quantified in the white matter (WM) and cell layers of both the lateral hemisphere (LH) and vermis (Ver) of the cerebellum. Increased gliosis, marked by GFAP⁺ immunoreactivity were seen in the WM lateral hemisphere and cell layers (B) and the cell layers only of the vermis (C). Increased microglial activation, marked by F4/80⁺ immunoreactivity, was seen in the white matter LH (D) and cell layers of the vermis (E). There was a decrease in F4/80⁺ immunoreactivity in the cell layers of the lateral hemisphere. Data presented as mean±SEM, asterisks indicates significance compared to control (**P*<0.01, ***P*<0.001, ****P*<0.001).

Fig. 2.

Activated Bergmann glia are found in close proximity to regions devoid of Purkinje cells in *Cln3*^{-/-} cerebellum. Anti-Calbindin labeling outlines the normal Purkinje cells morphology in the wild-type cerebellum with cell soma in the PCL (A) and radial processes spanning the cerebellar wall (A). Loss of *Cln3* within the cerebellum leads to a strong activation of GFAP within Bergmann glia (B–B', C–C') with clusters of GFAP⁺ Bergmann glia extending their reactive processes to the pial surface of the cerebellum (B, C; arrows). In the *Cln3* null mice, Purkinje cells within these regions of heavy GFAP⁺ gliosis are frequently absent or misplaced (B', C', asterisks). Immunolabeling with anti-MAP2 antibody, a microtubule associated protein expressed in neuronal dendrites and cell bodies (D, F) showed a similar *Cln3*^{-/-} selective loss of Purkinje cell within regions of GFAP⁺ activated Bergmann glia (E). Furthermore, anti-MAP2 immunolabeling revealed morphological changes in regions adjacent to GFAP⁺ activated Bergmann glia, including infiltration of a small diameter cell population into the PCL (E, G; arrowheads) and, within the ML, disorganization in the dendritic processes (G) compared to the organized pial orientation in the controls (F).

Fig. 3.

JNCL patient cerebella show glial activation and loss of Purkinje cells.

Immunohistochemistry of autopsy material from the cerebellum of JNCL patients showed loss of Purkinje cells and activation of astrocytes. Sections from neurologically normal control cases showed limited GFAP staining and a robust number of calbindin⁺ Purkinje cells (A). In JNCL patients (B, C), there was increased staining of GFAP⁺ processes (green) throughout the molecular layer, coinciding with regions devoid of calbindin⁺ (red) Purkinje cells. The relative abundance of GFAP immunoreactivity and the extent of Purkinje cell loss correlated qualitatively with disease severity, being less evident in a slowly progressing JNCL case (age at death 22 years, B) and more pronounced in a more rapidly progressing case (age at death 16 years, C) which closely resembled the appearance of the earlier onset late infantile form of NCL (age at death 6 years, D).

Fig. 4.

Misoriented Purkinje cells and an increase in dendritic spine number in *Cln3*^{-/-} mouse cerebellum. To observe the orientation of the Purkinje cells from the cerebellum of *Cln3*^{-/-} (A, right panel) and wild-type (A, left panel) mice, we stained sections with an anti-calbindin antibody followed by a DAB reaction. Purkinje cells in the *Cln3*^{-/-} cerebellum showed an altered orientation (A, black arrow). To quantitate the angle of the apical dendrite coming from the Purkinje cells, cerebellum from six month old *Cln3*^{-/-} and wild-type mice were subjected to Golgi impregnation. 100 μ m sagittal sections were cut, mounted, and developed from the cerebellum. The average angle of the apical dendrite to the pial surface was measured revealing a disruption in the angle in the *Cln3*^{-/-} mouse (B). Dendritic spine number was quantified as the number of Purkinje cell dendritic spines from six month old *Cln3*^{-/-} mice and wild-type cerebellum (C). The cerebella were separated into four regions (left and right lateral hemispheres and vermis) and labeled with an anti-calbindin antibody to visualize the Purkinje cells and their dendritic spines. There was a significant increase in the spine number in the left vermis from the cerebellum of *Cln3*^{-/-} mice ($N=3$ mice/genotype; five fields per section; five sections per region). Data shown are mean $\pm$ SEM, asterisks indicates significance compared to control (** $P<0.01$, *** $P<0.001$).

Fig. 5.

Selective loss of projection neurons in the medial deep cerebellar nuclei of *Cln3*^{-/-} mice. Cerebellar sections from six month old wild-type (A) and *Cln3*^{-/-} (B) mice were stained with cresyl violet and analyzed for difference in volume and cell number within the deep cerebellar nuclei (DCN) (L = lateral, I = intermediate, M = medial). Unbiased Cavalieri estimates of regional volume were measured on Nissl stained brain sections. Regional volumes were expressed in μm^3 and the mean volume of each region calculated for wild-type control and *Cln3*^{-/-} mice (C). No difference was seen in the volume of these nuclei. Nucleator measurements of cell area, expressed in μm^2 , were measured and no differences were seen in the area of the cells within the three nuclei (D). Optical fractionator estimates of the number of large projection neurons (E) or small cells which comprised of interneurons, glia, and endothelial cells (F) revealed a significant loss in the large projection neurons of the medial deep cerebellar nuclei (DCN) of *Cln3*^{-/-} mice compared with wild-type controls. There were no changes in the lateral or intermediate DCN. Data shown are mean $\pm$ SEM, asterisks indicates significance compared to control (* $P < 0.01$).

Fig. 6.

Alteration in cellular proliferation within the developing cerebellum of *Cln3*^{-/-} mice. (A) Measurements of the relative thickness of individual laminae of the early postnatal cerebellum (P7) reveal selective effects upon the internal granule cell layer (IGL) of *Cln3* mutant mice (*Cln3*^{-/-}) compared to wild-type controls with a significant reduction in the thickness of the IGL that contributes to the overall reduction in total cerebellar thickness (Total) evident in *Cln3*^{-/-} mice. In contrast, there were no significant effects upon the thickness of the external granule (EGL) or molecular layer (Mol) in these *Cln3* mutant mice. ****P*<0.001, one way ANOVA). (B, C) Cerebella from P6 *Cln3*^{-/-} and wild-type control mice were dissociated and separated on a Percoll gradient to isolate a population of cells which included glial cells and a lower fraction at the 35/60% interface which including proliferating granule neurons. Cells were pulsed with BrdU for one hour and processed for flow cytometry. The CGN fraction gave a significantly higher percentage of cells positive for the incorporation of BrdU in *Cln3*^{-/-} compared to wild-type (B). In the fraction containing high buoyancy cells, including astrocytes and endothelial cells, there were a significantly higher percentage of cells incorporating BrdU in the *Cln3*^{-/-} compared to wild-type (C). Differences were determined (Student's t-test, ****P*<0.001) and values are plotted ±SEM. Quantitative real-time PCR analysis was performed at various time points P2, P4, P5, and P7 (D). Normalized C_T values were calculated by averaging triplicate measurements of each sample (*N*=2). The change in C_T (ΔC_T) was calculated by subtracting the normalized C_T for the gene of interest (GOI) from the normalized C_T for the housekeeping (HK) gene (GAPDH). This relative expression of *Notch1*, *Notch2*, *Math1*, or *Hes1* in *Cln3*^{-/-} was compared to wild-type animal (Student's t-test, ****P*<0.001). (E) Fold differences were calculated by determining the change between wild-type and *Cln3*^{-/-} (ΔΔC_T=wild-type ΔC_T - *Cln3*^{-/-} ΔC_T for each sample) raised to the power of 2±SEM where the wild-type cell is set to 1 [any expression below 1 represents a decreased expression relative to wild-type] (Pfaffl, 2001).