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Neurodevelopmental delay in the *Cln3*$^{\Delta ex7/8}$ mouse model for Batten disease

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Juvenile neuronal ceroid lipofuscinosis (JNCL), also known as Batten disease, is a fatal inherited neurodegenerative disorder. The major clinical features of this disease are vision loss, seizures and progressive cognitive and motor decline starting in childhood. Mutations in *CLN3* are known to cause the disease, allowing the generation of mouse models that are powerful tools for JNCL research. In this study, we applied behavioural phenotyping protocols to test for early behavioural alterations in *Cln3*$^{\Delta ex7/8}$ knock-in mice, a genetic model that harbours the most common disease-causing *CLN3* mutation. We found delayed acquisition of developmental milestones, including negative geotaxis, grasping, wire suspension time and postural reflex in both homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ preweaning pups. To further investigate the consequences of this neurodevelopmental delay, we studied the behaviour of juvenile mice and found that homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ knock-in mice also exhibit deficits in exploratory activity. Moreover, when analysing motor behaviour, we observed severe motor deficits in *Cln3*$^{\Delta ex7/8}$ homozygous mice, but only a mild impairment in motor co-ordination and ambulatory gait in *Cln3*$^{\Delta ex7/8}$ heterozygous animals. This study reveals previously overlooked behaviour deficits in neonate and young adult *Cln3*$^{\Delta ex7/8}$ mice indicating neurodevelopmental delay as a putative novel component of JNCL.

Keywords: Batten, CLN3, disease models, genetics, mouse behaviour, neurodevelopment, neuronal ceroid lipofuscinosis

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Juvenile neuronal ceroid lipofuscinosis (JNCL; Batten or Spielmeyer–Vogt disease) is part of a family of fatal lysosomal storage disorders. Progressive visual impairment is typically the first clinical sign of this disease (Consortium 1995; Kohlschutter *et al.* 1993), followed by seizures, cognitive decline and motor dysfunction (Bennett & Hofmann 1999; Raininko *et al.* 1990). Premature death normally occurs around the third decade of life. These symptoms underscore the severe consequences of the autosomal recessive inheritance of mutations in *CLN3*. The vast majority of JNCL patients have a common 1.02 kb deletion in *CLN3* leading to the loss of exons 7 and 8 (Consortium 1995). *CLN3* encodes a 438 amino acid membrane protein with several proposed functions (Hobert & Dawson 2007; Holopainen *et al.* 2001; Koike *et al.* 2005; Narayan *et al.* 2006; Osorio *et al.* 2007; Pearce *et al.* 1999; Puranam *et al.* 1999; Ramirez-Montealegre & Pearce 2005), but our understanding of JNCL pathogenesis is still elusive. Four JNCL mouse models have been developed, namely the *Cln3*$^{\Delta ex1\text{-}6}$ knockout mouse (Mitchison *et al.* 1999), the *Cln3*$^{\Delta ex7/8}$ knockout (Katz *et al.* 1999), the *Cln3*$^{\Delta ex7/8}$ knock-in (Cotman *et al.* 2002) and the *Cln3*^{LacZ} β-galactosidase reporter model (Eliason *et al.* 2007). The *Cln3*$^{\Delta ex7/8}$ knock-in is the only model that recapitulates the most commonly observed 1.02 kb deletion, leading to the loss of exons 7 and 8 (Consortium 1995; Cotman *et al.* 2002). Transcript analysis in these mice showed that in addition to *Cln3* messenger RNA (mRNA) lacking exons 7 and 8, there were additional variant transcripts that lacked exon 5, or retained intron 1, 10 or 11, in combination with the loss of exons 7 and 8 (Cotman *et al.* 2002). The current sparse knowledge on JNCL pathological mechanism and CLN3 function limits our understanding on the possible relevance that variant *Cln3* transcripts might have. Therefore, the *Cln3*$^{\Delta ex7/8}$ knock-in mouse is a model tool of election for JNCL research. Presently, few efforts have been made in the behavioural characterization of *Cln3*$^{\Delta ex7/8}$ knock-in mice, and only gait and clasping behaviours have been evaluated in 10–12 months old animals (Cotman *et al.* 2002). As symptoms in JNCL emerge in childhood and several studies point to early initiation of JNCL disease process (Cotman *et al.* 2002; Herrmann *et al.* 2008; Kovacs *et al.* 2006; Lake 1993), special attention should be placed in characterizing behavioural alterations at early ages. In addition, the heterozygous *Cln3*$^{\Delta ex7/8}$ knock-in mice should also be characterized because mild alterations have also been reported in JNCL carriers (Gottlob *et al.* 1988; Sayit *et al.* 2002). Therefore, in the present study, we have applied behavioural paradigms

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suitable to study sensorial and motor capabilities in developing wild type, heterozygous and homozygous *Cln3*$^{\Delta ex7/8}$ knock-in preweaning pups. Furthermore, we analysed the behaviour of young adult animals using tests to assess major clinical features of JNCL, including alterations of selected behaviour profiles (open-field, elevated plus maze and forced swimming tests), visual function (visual cued version of Morris water maze test) and motor capacity (rotarod test and gate analysis).

Materials and methods

Animals

The strain used in this study was generated in the M. MacDonald laboratory (Cotman *et al.* 2002). The original strain was backcrossed 16 times to wild-type C57BL/6J mice obtained from Jackson laboratory. Mice were kept in an animal facility in a 12-h light : 12-h dark cycle (light onset at 0730 h), with food and water available *ad libitum*. Male and female heterozygous *Cln3*$^{\Delta ex7/8}$ animals were bred. A daily inspection for the presence of new litters in the cages was carried out twice a day, and the day of birth was annotated for each litter. After birth, animals were kept in the home cage with their mothers and were then tagged with non-toxic paint (green paste; Ketchum Manufacturing Inc., Brockville, Canada) in one or two toes per feet at PND 3. Pups were evaluated daily (wild type, $n = 25$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 33$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 29$ from 10 litters) in battery of test (approximately 8 min per animal) to evaluate somatic parameters and neurological reflexes until weaning at PND 21. At this point, the tip of the tail was cut for DNA extraction, and genotyping was performed by a multiplex polymerase chain reaction (PCR) analysis in a 20 μl volume that included autoclaved ultrafiltered water, PCR buffer (1×), dNTP mixture (200 μM each), primers (for details, see Table 1), Taq DNA polymerase (1 U/20 μl) and approximately 50 ng genomic DNA templates. PCR cycling conditions included 35 cycles of 15 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 65°C after a 10-min initial period of DNA denaturation and enzyme activation at 94°C. The amplified fragments had sizes readily distinguishable by electrophoresis through a 2% agarose gel. Groups of 8-week-old animals from nine litters that had not been previously tested in behaviour experiments were tested in different days in the open-field (wild type, $n = 24$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 27$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 25$), elevated plus maze (wild type, $n = 28$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 31$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 27$) and forced swimming tests (wild type, $n = 10$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 13$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 13$). Different groups of 8-week-old untested animals were used for rotarod, gait analysis and Morris water maze tests (wild type, $n = 9$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 12$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 15$). The same observer, blinded for animal genotype, evaluated each test performed. Tests were always made in the same circadian period and whenever possible at the same hour of the day. After completing the experiments, animals were euthanized by CO_2 and decapitated, thus minimizing their suffering. All animal experimentation was conducted in accordance with the European Community Council Directive, 86/609/EEC and National Institutes of Health (NIH) guidelines on animal care and experimentation.

Table 1: Primers used in multiplex PCR genotyping

Primer	Sequence 5′–3′	Concentration (nM)
Cln3 552F	GAG CTT TGT TCT GGT TGC CTT C	200
Cln3 Ex9R	GCA GTC TCT GCC TCG TTT TCT	200
Cln3 WTF	CAG CAT CTC CTC AGG GCT A	200
Cln3 WTR	CCA ACA TAG AAA GTA GGG TGT GC	200

Somatic parameters

Body weight and the anogenital distance were measured daily from PND 3 to PND 21. In addition, the day of eye opening and ear opening as well as fur appearance were also evaluated.

Neurological reflexes

Neurodevelopment testing for surface righting, air righting, wire suspension, negative geotaxis and postural reflexes was performed with minor alterations from what had been previously described (Mesquita *et al.* 2007; Santos *et al.* 2007). Briefly, animals were separated from their mother at the beginning of each test session and kept with their littermates in a new cage, under soft white light, with towel paper and sawdust from their home cage. The mothers were left in the same room as the pups during separation. Animals were returned to their home cage once testing was finished.

Surface righting reflex

The neonate was placed in the supine position, and the time needed to turn over and restore its normal prone position was recorded for a maximum of 30 seconds. Complete acquisition of the reflex was assumed when the animal could rotate 180° around its longitudinal axis.

Air righting reflex

The neonate was held on its back 30 cm above a soft surface before being released. The position in which the animals reach the soft pad was recorded. The reflex was considered to be achieved when neonate landed on the surface with all four paws.

Grasping and wire suspension test

A metal bar was suspended 30 cm above a soft surface. The animal was held, and its forepaws were allowed to touch the bar. Complete acquisition of grasping reflex was assumed when the animal was able to grasp the bar with both forepaws. The time the animal was able to hold on the bar using only its forepaws (wire suspension time) was also recorded for a maximum of 30 seconds.

Negative geotaxis

The animal was placed on a grid, tilted 45° to the plane, with its head facing downwards. Animals that could rotate a full 180° and face up within a maximum time of 30 seconds were considered to have acquired this reflex.

Postural reflex

Neonates were placed in a 15 × 15 cm box and shaken left and right and up and down. Animals that could maintain their original position in the box by extending all four limbs were assumed to have acquired this skill.

Open field

Animals were placed in the centre of a 43.2 × 43.2 cm arena with transparent walls (MedAssociates Inc., St Albans, VT, USA) and were observed for 5 min. The arena was lighted by a 60 W bulb suspended above the centre. Activity parameters were automatically collected by the equipment (total distance travelled, speed, resting time, distance travelled and time spent in a predefined 10.8 × 10.8 cm square in the centre of the arena). The number of rears and the time that animals spent exploring vertically were registered by the observer.

Rotarod

Mice were tested in a rotarod apparatus from TSE systems (Hamburg, Germany). The protocol consisted of 3 days of training at the

constant speed of 15 r.p.m. for a maximum of 60 seconds in four trials, with a 15-min interval between each trial. At the fourth day, animals were tested for each of six velocities (5, 8, 15, 20 and 24 r.p.m.) for a maximum of 60 seconds in two trials, with a 10-min interval between each trial. The latency to fall off the rod was registered.

Elevated plus maze

Mice were placed in an Elevated plus maze (EPM) apparatus consisting of two opposite open arms (50.8 × 10.2 cm) and two opposite closed arms (50.8 × 10.2 × 40.6 cm) raised 72.4 cm above the floor (ENV-560; MedAssociates Inc.), and the time spent in each of the arms was measured using a video-tracking system (Viewpoint, Champagne au Mont d'Or, France).

Forced swimming test

Learned helplessness, as a measure of susceptibility to depression-related behaviour, was assessed using the forced swimming test. Mice were placed in cylinders (diameter: 37 cm; 55 cm of height) filled with water (25°C) to a depth where the animals had no solid support for their rear paws. After a 10-min pretest session, animals were rested for 24 h before being subjected to the actual tests, which lasted 5 min. At the end of each test session, animals were placed on a heating pad (15 min) before being returned to their home cages. Cylinders were filled with fresh water after each trial. A video camera, placed at the top of the cylinder, was used to record test sessions. Recordings were later scored by an investigator blinded to the experimental details to determine inactivity (passiveness – defined as time spent either immobile or making righting movements to stay afloat) vs. activity periods.

Gait analysis

Eight-week-old mouse were placed in an apparatus that consisted of a wooden platform 90 cm long × 9 cm wide with a wooden box (27 × 22 cm; 17.5 cm height) at one end of the platform; mice had free access to the box from the platform through a small door. The entire apparatus was elevated 11 cm from the bench, and paper (9 × 90 cm long) was placed on the walking platform; the hind paws of the mouse were dipped into non-toxic black paint and the forepaws were painted with red paint. In the day of testing, individual mice were placed on the end of the platform farthest from the box. As each mouse traversed the platform, its gait was recorded as finger-paint paw prints. Several parameters of the gait were measured, including length of stride (distance between consecutive same footprints), length of step (distance between consecutive alternate footprints) and hind and forepaws displacement (distance between left and right hind or fore paws).

Morris water maze with visual clues

The Morris water maze consisted of a black tank (diameter: 170 cm, depth: 50 cm), divided into four quadrants by virtual lines and filled with water (22°C) to a depth of 31 cm. During testing, a visible platform (12 × 12 cm; identified with a 10 cm^2 square flag with 15 cm of height) was placed at a height of 30 cm, and extrinsic visual clues were glued to the walls. Data were collected using a video-tracking system (Viewpoint, Champagne au Mont d'Or, France). Animals were tested for three consecutive days (four trials per day, with a maximum of 2 min per trial). The visible escape platform was placed in the centre of an arbitrarily defined quadrant. Test sessions began with mice being placed, facing the wall of the maze, in a defined start position and finished once the escape platform had been reached. This procedure was continued in a clockwise fashion over the subsequent trials. The time and distance to escape to the platform were recorded. In cases in which the escape platform had not been reached within 2 min, the experimenter guided the animal to the platform. In either case, animals were dried and allowed to rest for 30 seconds before being returned to the maze for the remaining test sessions.

Quantitative reverse transcription polymerase chain reaction

For comparative gene expression studies, total RNA was extracted from the cerebellum isolated from wild type, heterozygous and homozygous *Cln3*$^{\Delta ex7/8}$ mice using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated using the Turbo™ DNA-free kit (Ambion, Austin, TX, USA) to reduce genomic DNA contamination. One microgram of RNA was used as template for complementary DNA (cDNA) synthesis using the High-Capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. A total of three mice per genotype were used for reverse transcription polymerase chain reaction (qRT-PCR) analysis. Comparative quantitative PCR was performed using gene-specific primers as detailed in Table 1 using β-actin for normalization. Amplification was carried out using Power SYBR Green master mix (Applied Biosystems) containing appropriate concentrations of each primer (Table 2) and 2 μl of cDNA in a 96 well plate on a Mx3005p real-time PCR instrument (Stratagene, La Jolla, CA, USA) using the following cycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 20 seconds and 60°C for 1 min. Specificity of the amplified product was determined by melt-curve analysis immediately following completion of the final amplification cycle.

Statistical analysis

For each component of the test battery for physical maturation and neuronal reflex acquisition, the average first day of adult-like responding was statistically analysed by analysis of variance (ANOVA), with Tukey multiple comparison procedure. Further analysis of these data was performed in different PND by comparing the percentage of animals with and without adult-like response against two genotypes (wild type vs. heterozygous and wild type vs. homozygous *Cln3*$^{\Delta ex7/8}$ mice) by the Fisher's exact test (FET). Other statistical comparisons were performed, in the case of two groups, through Student's *t*-test or, in the case of several groups, by ANOVA, with Tukey multiple comparison procedure. When the homogeneity of variances was not observed, non parametric tests were used, Mann–Whitney test for two groups and Kruskal–Wallis test for several groups, with Bonferroni correction for multiple comparisons. Relative gene expression and statistical analysis of qRT-PCR data were calculated using the REST-XL version 2 program (Pfaffl *et al.* 2002) and expressed as fold change vs. wild type. Data from male and female animals were pooled together when there was no sex effect and sex vs. genotype interactions as

Table 2: Primers used in qRT-PCR

Primer		Sequence 5′–3′	Concentration (nM)
Set 1	Cln3 ex1F	TGA GAG GGA GGA GAC CGA CTC AGA	400
	Cln3 ex3R	CCA AGA TCC AGA AAC CCA CTG CA	400
Set 2	Cln3 ex6F	TCT GGT TGC CTT CTC TCA GTC AGT	300
	Cln3 ex7/8R	AGA CCA CCA TGA GAT CAC AGC ACT	300
HSK	ActB ex1F	CTG TCG AGT CGC GTC CAC CC	600
	ActB ex2R	CGT CAT CCA TGG CGA ACT GG	600

verified by the two-way ANOVA test. In rotarod experiments, homogeneity of variances was not observed (Levene's test), and only male animals were used. SPSS version 16 was used to analyse the data, and statistical significance was set to $P < 0.05$.

Results

Preweaning neurodevelopment abnormalities in Cln3$^{\Delta ex7/8}$ neonates

To evaluate the possible influence of *Cln3*$^{\Delta ex7/8}$ mutation in the appearance of developmental milestones, we performed daily analysis/scoring of physical growth, maturation and acquisition of neurological reflexes in wild type, homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ knock-in littermates from PND 3 to 21. Both homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ knock-in exhibited delays in the achievement of developmental milestones (Table 3). The greatest delays were observed in the negative geotaxis and grasping reflex. The mean first day of appearance on negative geotaxis reflex was significantly different among the tree genotypes ($F_{2,71} = 3.180$, $P < 0.05$) and delayed in approximately 3.0 days for homozygous *Cln3*$^{\Delta ex7/8}$ mice and 2.2 days for the heterozygous *Cln3*$^{\Delta ex7/8}$ mice. In the grasping behaviour, there were also significant differences between the genotypes ($F_{2,72} = 3.024$, $P < 0.05$), and the delay was of approximately 2.5 days for the homozygous *Cln3*$^{\Delta ex7/8}$ mice and 1.3 days for the heterozygous *Cln3*$^{\Delta ex7/8}$ mice. The differences in the mean first day of adult-like response did not reach statistical significance for the other tests performed. Nonetheless, when analysing the percentage of animals with adult-like response at the different PNDs, in addition to finding significant differences in negative geotaxis at PND 5 and 6 (FET, $P < 0.05$) and in grasping at PND 6 (FET, $P < 0.05$), we also found significant differences among genotypes in the percentage of animals with postural reflex at PND 8 (FET, $P < 0.05$) (Fig. 1a). Furthermore, the time of wire suspension was also significantly decreased ($F_{2,50} = 3.748$, $P < 0.05$) in homozygous *Cln3*$^{\Delta ex7/8}$ at PND 16 when compared with wild-type controls (Fig. 1b). These results indicate the presence of a delay in the neurodevelopment of homozygous *Cln3*$^{\Delta ex7/8}$ mice that is less severe but also present in heterozygous animals.

Table 3: Developmental milestones of wild type, homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ knock-in neonatal mice

	Wild type	*Cln3*$^{+/\Delta ex7/8}$	*Cln3*$^{\Delta ex7/8/\Delta ex7/8}$
Air righting reflex, first day	14.78 ± 0.90	15.32 ± 0.92	15.87 ± 1.23
Surface righting reflex, first day	4.08 ± 0.90	4.62 ± 1.19	4.93 ± 1.46
Eye opening, first day	12.30 ± 3.34	13.33 ± 1.19	13.48 ± 1.45
Ear opening, first day	13.07 ± 0.47	13.18 ± 0.51	13.00 ± 0.35
Negative geotaxis, first day	4.50 ± 0.85	6.71 ± 2.78	7.52 ± 3.38
Postural reflex, first day	8.13 ± 0.51	8.75 ± 2.13	8.70 ± 1.96
Grasping, first day	5.13 ± 0.58	6.40 ± 0.59	7.60 ± 0.57

Data are presented as mean first day of adult-like response ± SE (wild type, $n = 25$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 33$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 29$).

Juvenile Cln3$^{\Delta ex7/8}$ knock-in mice display reduced exploratory behaviour

Having observed neurodevelopmental delays in *Cln3*$^{\Delta ex7/8}$ mice, we sought to evaluate the possible consequences of this anomaly in the behaviour of postweaning 8-week-old animals in the open-field test. A marked decrease in the exploratory behaviour of homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ mice, expressed by a significant decrease in the number of 'rearings' performed ($F_{2,73} = 7.193$, $P < 0.05$) (Fig. 2a) and time spent in vertical exploration ($F_{2,73} = 7.300$, $P < 0.05$) (Fig. 2b), was found. Spontaneous locomotion was not affected because no genotype-associated differences were found in the total distance travelled (Fig. 2c). Interestingly, there were significant differences in the percentage of time heterozygous or homozygous *Cln3*$^{\Delta ex7/8}$ mice ($F_{2,73} = 5.926$, $P < 0.05$) spent in the centre of the arena when compared with wild-type animals (Fig. 2d). As this could be indicative of anxiety-like behaviour, we then tested the animals in the elevated plus maze test. There were no significant differences neither in the percentage of time spent in the open arms (Table 4a) nor in the number of open arms entries (data not shown), indicating no evidence for anxiety-like behaviour. This result suggests that the decrease in the percentage of time spent in the centre of the field by *Cln3*$^{\Delta ex7/8}$ knock-in mice is a consequence of the decreased exploratory activity observed in these animals. Finally, the forced swimming test gave no indications of depressive-like behaviour because no differences were observed between the different genotypes neither in the percentage of time spent in immobility status (Table 4b) nor in the latency to immobility time (data not shown).

Juvenile Cln3$^{\Delta ex7/8}$ knock-in mice do not have marked visual impairment

Visual impairment is typically the first clinical sign detected in JNCL human patients (Kohlschutter *et al.* 1993). Therefore, we performed an analysis of visual acuity in 8-week-old wild-type, heterozygous and homozygous *Cln3*$^{\Delta ex7/8}$ animals. For this purpose, we used a modified version of the Morris water maze with visual clues to indicate the platform. Testing was performed over 3 days (four trials per day), and our results show no significant genotype-associated differences neither in time needed (Table 4c) nor in the distance swam to escape to the visible platform (data not shown).

Juvenile Cln3$^{\Delta ex7/8}$ mice show motor co-ordination impairment and ataxia

Motor deficits have been established as one of the primary clinical features in JNCL emerging early in disease progression (Raininko *et al.* 1990). To evaluate motor co-ordination,

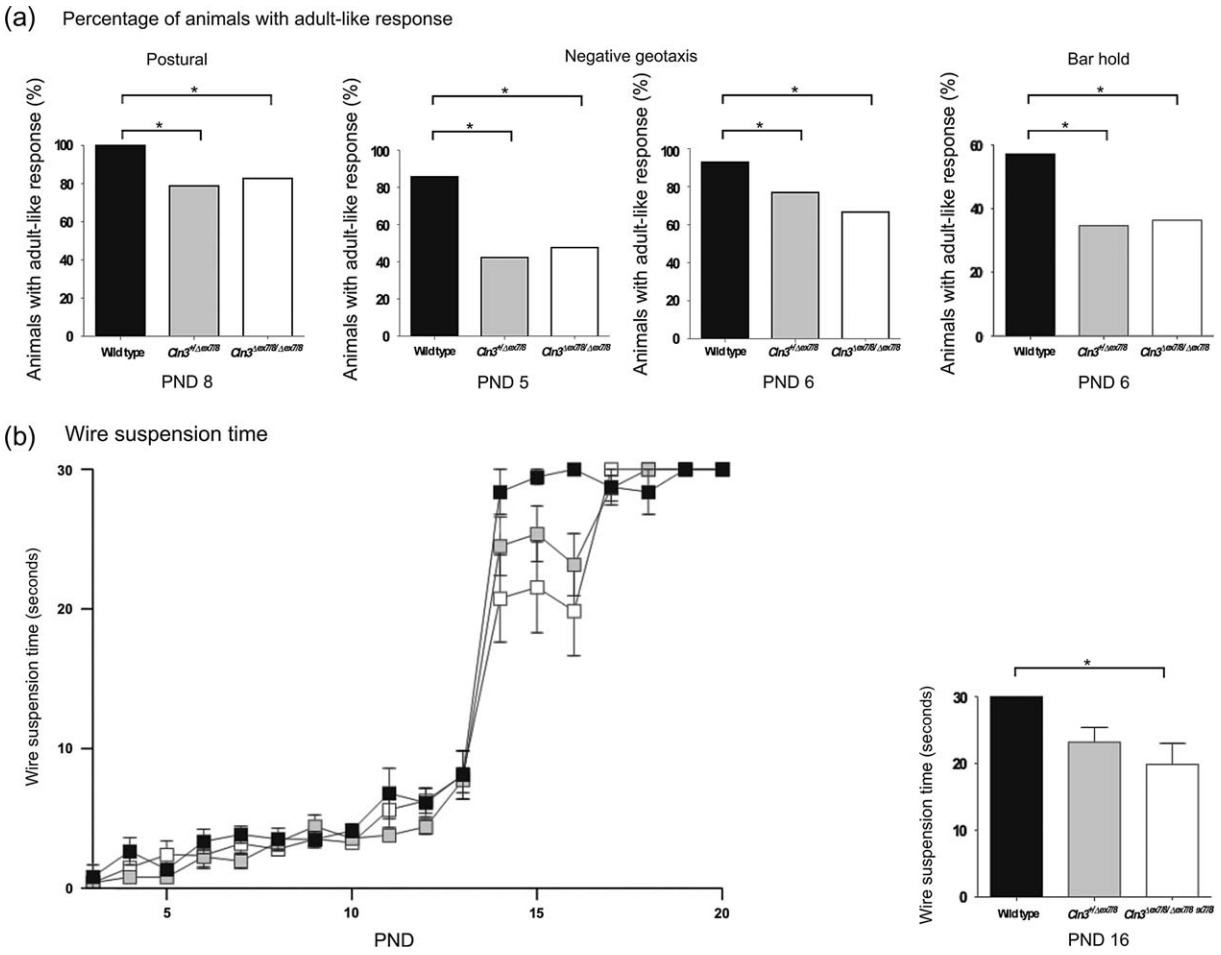

Figure 1: Decreased percentage of animals with negative geotaxis, postural reflex and grasping behaviour in homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$. (a) The percentage of homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ mice positively scored was significantly decreased for postural reflex at PND 8, for negative geotaxis at PND 5 and 6 and for grasping behaviour at PND 6. Data are expressed as the percentage of animals achieving adult-like response (b) The time of wire suspension was also significantly decreased in homozygous *Cln3*$^{\Delta ex7/8}$ at PND 16 when compared with wild-type controls. Mean values are plotted with SEM. *P<0.005. (wild type, $n = 25$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 33$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 29$).

we started measuring the latency time required by 8-week-old wild-type, heterozygous and homozygous *Cln3*$^{\Delta ex7/8}$ knock-in mice to fall from rods rotating at the speed of 5, 8, 15, 20, 24 and 31 r.p.m. (Fig. 3a). Mean latency times were significantly different between groups at the speeds of 15, 20 and 24 (Kruskal–Wallis test, $\chi^2_{(2)} = 11.379, 9.254, 8.058$, respectively, $P < 0.05$). *Cln3*$^{\Delta ex7/8}$ homozygous mice present significantly decreased latency times to fall at the speed of 15 ($z = -2.246$, $P < 0.05$), 20 ($z = -2.453$, $P < 0.05$) and 24 r.p.m. ($z = -2.565$, $P < 0.05$) when compared with the wild-type controls. *Cln3*$^{\Delta ex7/8}$ heterozygous mice only have significantly decreased latency time to fall from the rod when compared with wild-type controls at the speed of 24 r.p.m. ($z = -2.380$, $P < 0.05$). We also analysed the ambulatory gait of *Cln3*$^{\Delta ex7/8}$ mice. *Cln3*$^{\Delta ex7/8}$ homozygous mice displayed decreased displacement between hind paws ($q = 2.614$, $P < 0.05$) (Fig. 3b) and forepaws ($q = 2.414$, $P < 0.05$) (Fig. 3c) when compared with wild-type controls, while presenting no stride length or stride matching differences (data not shown), which is indicative of mild ataxia. *Cln3*$^{\Delta ex7/8}$ heterozygous only showed decreased displacement in the hind paws ($q = 4.106$, $P < 0.05$) (Fig. 3b). Collectively, these results indicate motor deficits in 8-week-old *Cln3*$^{\Delta ex7/8}$ homozygous mice and less severe motor deficits in heterozygous *Cln3*$^{\Delta ex7/8}$ mice.

Heterozygous Cln3$^{\Delta ex7/8}$ *mice express intermediate levels of wild-type and mutant Cln3 mRNA*

Comparative qRT-PCR was used to determine the relative expression of *Cln*3 mRNA transcripts in wild-type, heterozygous and homozygous *Cln3*$^{\Delta ex7/8}$ mice. Previously, it was shown that *Cln3*$^{\Delta ex7/8}$ mice express multiple variant transcripts in response to the deletion of exons 7 and 8 from the genomic sequence (Cotman *et al.* 2002). Because

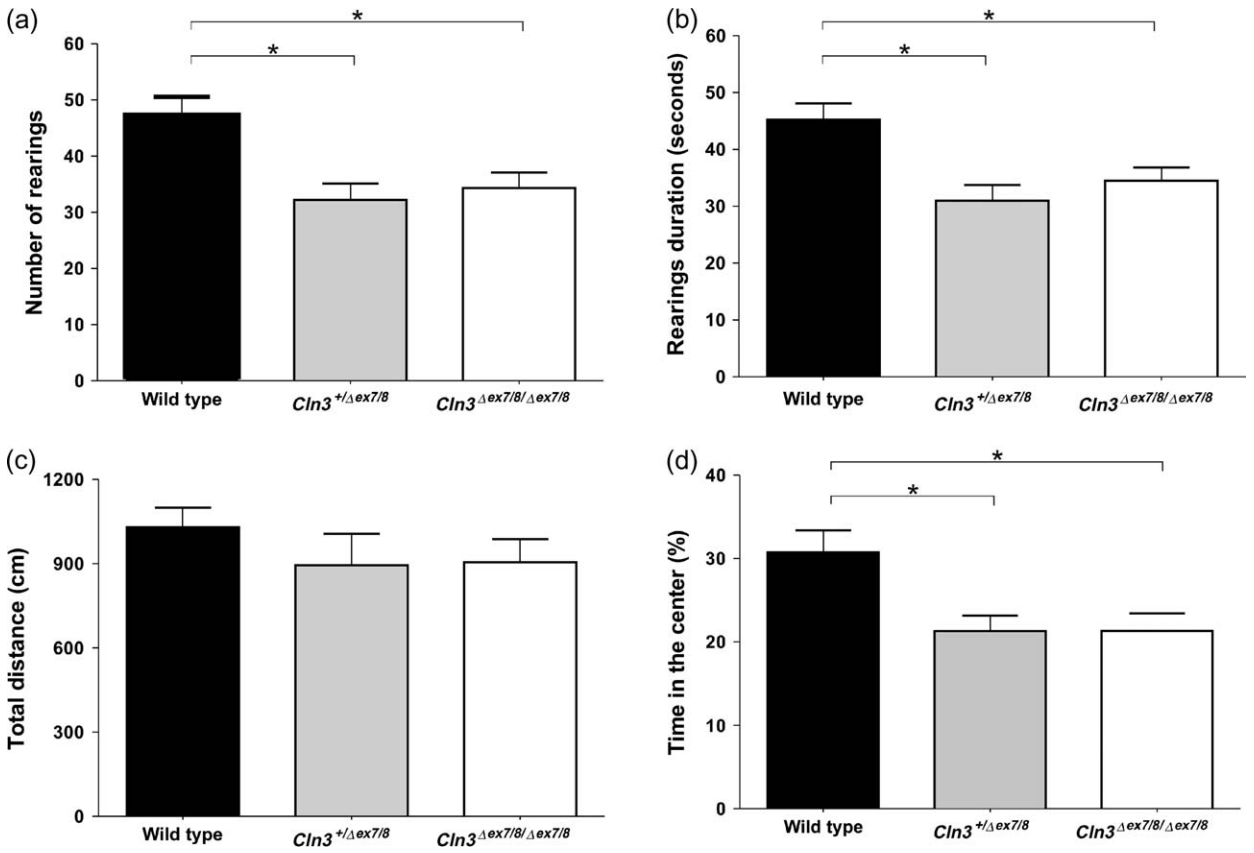

Figure 2: Decreased exploratory activity in homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ mice. Eight-week-old animals were tested in the open-field apparatus. Homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ mice have a significant decrease in (a) the number of 'rearings' performed and in (b) the total time spent in vertical exploration. (c) The total distance *Cln3*$^{\Delta ex7/8}$ mouse travelled during the test is normal while presenting a significant decrease in (d) the percentage of time spent in the centre of the field. Mean values are plotted with SEM, *P<0.05 (wild type, $n = 24$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 27$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 25$).

heterozygote animals have one wild-type allele and one mutant allele, it is assumed that these mice would express partial transcripts lacking exons 7 and 8 (mutant transcript) in addition to the normal full-length transcript. To distinguish between full-length wild-type and mutant transcripts, primers were designed to detect either all *Cln3* transcripts or only full-length wild-type transcripts. Primer set 1 was designed to amplify a region within exon 1 through exon 3 that is upstream of the deleted region and thus is common to both wild-type and mutant *Cln3* transcripts. Primer set 2 amplifies a region within exon 6 through exon 8 and thus would detect only full-length *Cln3* transcripts because mutant transcripts

Table 4: Behaviour of wild type, homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ knock-in 8-month-old mice in the elevated plus maze (wild type, $n = 28$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 31$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 27$) (a), forced swimming (wild type, $n = 10$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 13$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 13$) (b) and visual cued water maze tests (wild type, $n = 9$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 12$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 15$) (c)

	Wild type	*Cln3*$^{+/\Delta ex7/8}$	*Cln3*$^{\Delta ex7/8/\Delta ex7/8}$
(a) Elevated plus maze: time in open arms (%) (mean ± SEM)	14.43 ± 2.617	13.80 ± 2.125	13.25 ± 2.444
(b) Forced swimming: immobility time (%) (mean ± SEM)	59.48 ± 8.531	75.76 ± 2.364	65.71 ± 4.192
(c) Visual cued water maze: escape latency time (seconds) (mean ± SEM)			
Day 1	71.83 ± 6.306	82.58 ± 6.345	72.48 ± 5.861
Day 2	33.15 ± 5.281	35.58 ± 5.048	30.71 ± 3.879
Day 3	27.11 ± 5.285	20.81 ± 2.832	16.20 ± 2.222

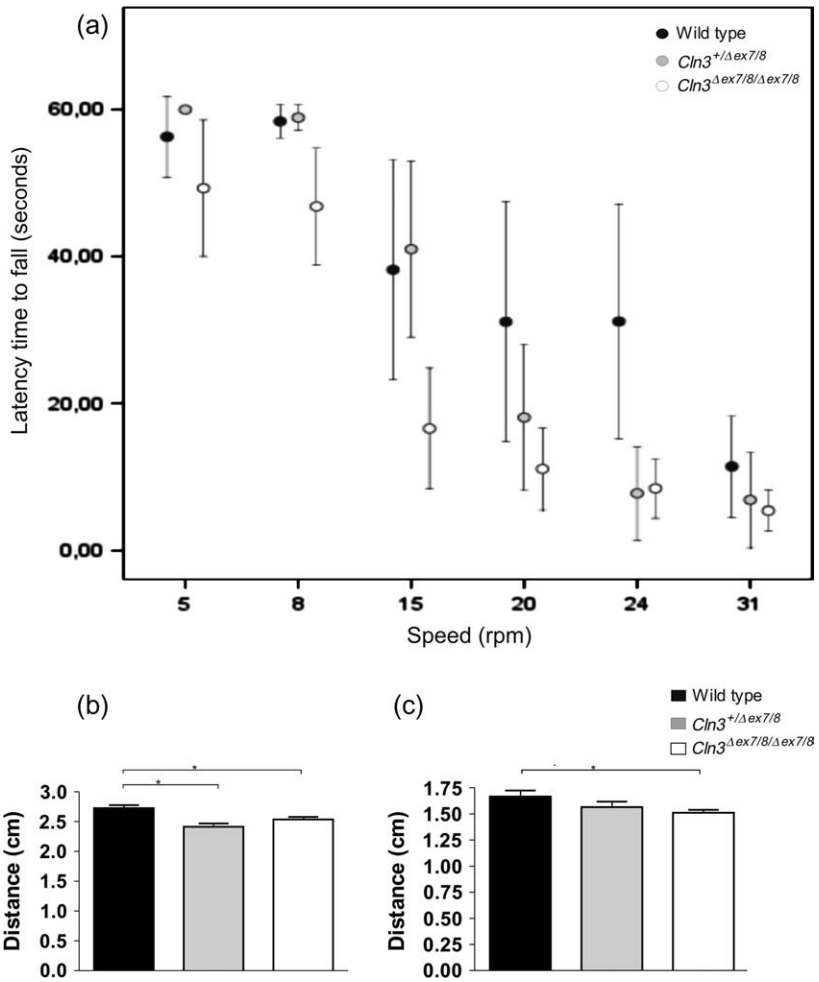

Figure 3: Evidence for motor deficits in homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ mice. (a) When tested in the rotarod, homozygous 8-week-old *Cln3*$^{\Delta ex7/8}$ mice have a significantly decreased latency to fall off the rod at the speeds of 15, 20 and 24 r.p.m., while heterozygous *Cln3*$^{\Delta ex7/8}$ mice only exhibit decreased latency to fall at the speed of 24 r.p.m. (wild type, $n = 11$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 13$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 17$). Mean values are plotted with confidence interval at a 95% level. Ambulatory gait was also analysed, and homozygous *Cln3*$^{\Delta ex7/8}$ mice have a significantly decreased displacement of (b) hind and (c) forepaws, while heterozygous *Cln3*$^{\Delta ex7/8}$ animals only present a decrease in (b) hind paws displacement (wild type, $n = 9$; heterozygous *Cln3*$^{\Delta ex7/8}$, $n = 12$; homozygous *Cln3*$^{\Delta ex7/8}$, $n = 15$). Mean values are plotted with SEM, $P < 0.05$.

lack exons 7 and 8. Using primer set 1, which detects both full-length and mutant *Cln3* transcripts, we observed no significant differences in *Cln3* transcript levels in heterozygote mice in comparison to wild type, but a 3.6-fold decrease ($P < 0.05$) in transcript levels in the homozygous mice. Because no changes in the overall level of *Cln3* was detected in heterozygotes, we sought to determine the relative contribution of full-length transcripts to the overall transcript level by using primer set 2, which is directed at the deleted region of *Cln3* such that mutant transcripts lacking exons 7 and 8 are not detected. Using this approach, we observed a 1.8-fold decrease ($P < 0.05$) in full-length transcripts in heterozygotes and no detectable transcripts in homozygote animals. These results suggest that in heterozygote mice, approximately half of the transcripts present are full length, with the remainder being mutant variant transcripts lacking exons 7 and 8.

Discussion

The use of the *Cln3*^{lacZ} reporter mouse model has shown that *Cln3* is expressed in the brain during embryonic and early postnatal stages (Eliason *et al.* 2007), further supporting early initiation of JNCL disease process (Cotman *et al.* 2002; Herrmann *et al.* 2008; Kovacs *et al.* 2006; Lake 1993). In addition, it has been suggested that mutated polypeptides resulting from expression of *Cln3*$^{\Delta ex7/8}$, the most common JNCL mutation, may have biological function (Kitzmuller *et al.* 2008). Therefore, we studied the early postnatal behaviour of heterozygous and homozygous *Cln3*$^{\Delta ex7/8}$ knock-in mice. In

homozygous *Cln3*$^{\Delta ex7/8}$ mice, we detected a significant delay in the acquisition of negative geotaxis and grasping reflexes and also a decrease in the percentage of animals with postural reflex at PND 8 and in the wire suspension time at PND 16. Interestingly, we also detected alterations in heterozygous *Cln3*$^{\Delta ex7/8}$ mice, including a less pronounced delay in the achievement of negative geotaxis and grasping reflex and a decrease in the percentage of animals with postural reflex at PND 8. These tasks evaluate vestibular and motor functions (Dierssen *et al.* 2002; Lake 1993), and our results suggest that *Cln3*$^{\Delta ex7/8}$ induces a delay in the maturation of brainstem and cerebellar structures not only in homozygous but also in heterozygous *Cln3*$^{\Delta ex7/8}$ mice. This is the earliest description of behavioural abnormalities in JNCL mouse models indicating delayed neurodevelopment as a putative novel component of JNCL disease. Adult behaviour phenotypes can be influenced by the deficit in one or more functional domains during neurodevelopment. In fact, it is possible that the detected developmental alterations are in the basis of the reduced exploratory activity encountered in both homozygous and heterozygous *Cln3*$^{\Delta ex7/8}$ young adult mice. When focusing the behavioural characterization in tests that assess the major and earlier clinical features of JNCL, we found no evidence for visual impairment in young adult *Cln3*$^{\Delta ex7/8}$ mice. Visual loss is, commonly, the earliest symptom detected in patients and has been associated with retinal degeneration (Goebel 1977), but the use of the visual cued version of Morris water maze pointed to the absence of marked visual impairment in *Cln3*$^{\Delta ex7/8}$ mice at juvenile age. This result is in accordance with previous reports showing minimal photoreceptor loss (Cotman *et al.* 2002; Seigel *et al.* 2002), normal electroretinograms (Seigel *et al.* 2002) and alterations in pupillary reflexes only in 24-month-old animals (Katz *et al.* 2008). Motor deficits are another major clinical feature of JNCL with early onset, which is mainly attributed to cerebellar degeneration (Raininko *et al.* 1990). In accordance with data from other JNCL mouse models (Eliason *et al.* 2007; Kovacs *et al.* 2006), 8-week-old homozygous *Cln3*$^{\Delta ex7/8}$ mice exhibit a marked motor impairment that is evident in the rotarod test. Interestingly, *Cln3*$^{\Delta ex7/8}$ heterozygous animals show a milder phenotype only detectable with increasing difficulty of the test. Additionally, previous studies also showed that 10- to 12-month-old homozygous *Cln3*$^{\Delta ex7/8}$ mice exhibit alterations in ambulatory gait (Cotman *et al.* 2002). Herein, we show that gait alterations are already present in 8-week-old homozygous *Cln3*$^{\Delta ex7/8}$ mice and are, again, present and milder in the heterozygous *Cln3*$^{\Delta ex7/8}$ mice. As the rotarod and gait analysis are highly influenced by cerebellar function, these behavioural results suggest that, as expected, homozygous *Cln3*$^{\Delta ex7/8}$ mice might undergo more pronounced cerebellar degeneration. The demonstration of behaviour deficits in heterozygous *Cln3*$^{\Delta ex7/8}$ mice is a novel finding of the present work because previous studies with the various JNCL murine models had never compared wild-type, heterozygous and homozygous animals (Cotman *et al.* 2002; Eliason *et al.* 2007; Katz *et al.* 2008; Mitchison *et al.* 1999). It has been reported that *Cln3*$^{\Delta ex7/8}$ knock-in mice exhibit earlier and more severe neurological disease (Cotman *et al.* 2002) when compared with *Cln3*^{LacZ} and *Cln3*$^{\Delta ex1-6}$ mouse models. This observation could be suggestive that variant *Cln3* transcripts might contribute to the pathological alterations. Nonetheless, the studies that support a more severe phenotype in *Cln3*$^{\Delta ex7/8}$ knock-in mice might be influenced by strain background effects and genetic modifiers because different strains were used (Cotman *et al.* 2002; Eliason *et al.* 2007; Katz *et al.* 2008; Mitchison *et al.*, 1999). This hypothesis is supported by the comparison of *Cln3*$^{\Delta ex7/8}$ knock-in mice with the *Cln3*$^{\Delta ex7/8}$ described by Katz *et al.* In these transgenic strains, exons 7 and 8 are deleted, albeit using differing strategies and targeted regions would result in a predicted truncated protein without exons 7 and 8. The *Cln3*$^{\Delta ex7/8}$(Katz) model exhibited a later onset of pathological changes (Katz *et al.* 1999, 2008) in a time frame more consistent with the *Cln3*$^{\Delta ex1-6}$ and *Cln3*^{LacZ} mice. This illustrates the need to perform a comprehensive characterization of the available murine JNCL models on a standard genetic background to allow proper comparison. We analysed the transcripts present in *Cln3*$^{\Delta ex7/8}$ mice and found intermediate levels of wild-type and truncated *Cln3* transcripts in heterozygous animals. A recent study suggests that the truncated CLN3 protein is unlikely to be expressed (Chan *et al.* 2008). Therefore, it is likely that wild-type CLN3 levels in heterozygous *Cln3*$^{\Delta ex7/8}$ animals are insufficient to maintain the required CLN3 activity resulting in the anomalies found. As previously suggested, a threshold for CLN3 activity might exists before the onset of neurological disease (Cotman *et al.* 2002). Nevertheless, at this point, we cannot fully discard a possible influence of alternative *Cln3* transcripts on the observed phenotype, and further studies at the biochemical and molecular levels are necessary to increase our understanding on this aspect. Irrespective of the underlying mechanism, it is interesting to note that existing studies in human patients have documented brain structural abnormalities and functional ophthalmological changes in JNCL carriers (Gottlob *et al.* 1988; Sayit *et al.* 2002), suggesting that heterozygous human carriers may also exhibit mild neurological alterations.

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