Neurodevelopmental delay in the Cln3<sup>Δex7/8</sup> 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<sup>Δex7/8</sup> 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<sup>Δex7/8</sup> 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<sup>Δex7/8</sup> knock-in mice also exhibit deficits in exploratory activity. Moreover, when analysing motor behaviour, we observed severe motor deficits in Cln3<sup>Δex7/8</sup> homozygous mice, but only a mild impairment in motor co-ordination and ambulatory gait in Cln3<sup>Δex7/8</sup> heterozygous animals. This study reveals previously overlooked behaviour deficits in neonate and young adult Cln3<sup>Δex7/8</sup> mice indicating neurodevelopmental delay as a putative novel component of JNCL.

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

<sup>1</sup>These authors contributed equally to this work.

suitable to study sensorial and motor capabilities in developing wild type, heterozygous and homozygous \textit{Cln3}^{\text{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 07:30 h), with food and water available \textit{ad libitum}. Male and female heterozygous \textit{Cln3}^{\text{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 \textit{Cln3}^{\text{ex7/8}}, \(n = 33\); homozygous \textit{Cln3}^{\text{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 \(\mu\)l volume that included autoclaved ultrafiltered water, PCR buffer (1x), dNTP mixture (200 \(\mu\)M each), primers (for details, see Table 1), Taq DNA polymerase (1 U/20 \(\mu\)l) and approximately 50 ng genomic DNA templates. PCR cycling conditions included 35 cycles of 15 seconds at 94\(^\circ\)C, 30 seconds at 55\(^\circ\)C and 45 seconds at 65\(^\circ\)C after a 10-min initial period of DNA denaturation and enzyme activation at 94\(^\circ\)C. The amplified fragments had sizes readily readable 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 \textit{Cln3}^{\text{ex7/8}}, \(n = 27\); homozygous \textit{Cln3}^{\text{ex7/8}}, \(n = 25\)), elevated plus maze (wild type, \(n = 28\); heterozygous \textit{Cln3}^{\text{ex7/8}}, \(n = 31\); homozygous \textit{Cln3}^{\text{ex7/8}}, \(n = 27\)) and forced swimming tests (wild type, \(n = 10\); heterozygous \textit{Cln3}^{\text{ex7/8}}, \(n = 13\); homozygous \textit{Cln3}^{\text{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 \textit{Cln3}^{\text{ex7/8}}, \(n = 12\); homozygous \textit{Cln3}^{\text{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/EC and National Institutes of Health (NIH) guidelines on animal care and experimentation.

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

Neurodevelopmental 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\(^\circ\) 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 with mid and forepaw on the bar was considered to have reached 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 forepaw was also recorded for a maximum of 30 seconds.

Negative geotaxis

The animal was placed on a grid, tilted 45\(^\circ\) to the plane, with its head facing downwards. Animals that could rotate a full 180\(^\circ\) 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 \(\times\) 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 \(\times\) 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 \(\times\) 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

Table 1: Primers used in multiplex PCR genotyping

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Concentration (nM)</th>
</tr>
</thead>
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<tr>
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<td>GAG CTT GAG GTG TGC</td>
<td>200</td>
</tr>
<tr>
<td>Cln3 ExBR</td>
<td>GCA GTC GCT TGG TCT</td>
<td>200</td>
</tr>
<tr>
<td>Cln3 WTR</td>
<td>CCA ACA TAG AAA GTA GGG</td>
<td>200</td>
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</tbody>
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Early behavioural alterations in Cln3<sup>Aex7/8</sup> mice

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 square flag with 15 cm of height) was placed at a height of 30 cm, and intrinsically 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<sup>Aex7/8</sup> 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<sup>Aex7/8</sup> 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 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>
<thead>
<tr>
<th>Primer</th>
<th>Concentration (nM)</th>
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<tr>
<td>TGA GAG GGA GGA</td>
<td>400</td>
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<tr>
<td>CCA AGA CTC AGA</td>
<td>400</td>
</tr>
<tr>
<td>TCT GGT TGC CTT</td>
<td>300</td>
</tr>
<tr>
<td>AGA CCA CCA TGA</td>
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<td>600</td>
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<tr>
<td>CGA ACT GG</td>
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</tbody>
</table>
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<sup>ex7/8</sup> neonates**

To evaluate the possible influence of Cln3<sup>ex7/8</sup> 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<sup>ex7/8</sup> knock-in littermates from PND 3 to 21. Both homozygous and heterozygous Cln3<sup>ex7/8</sup> 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 and grasping 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<sup>ex7/8</sup> mice and 2.2 days for the heterozygous Cln3<sup>ex7/8</sup> 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<sup>ex7/8</sup> mice and 1.3 days for the heterozygous Cln3<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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<sup>ex7/8</sup> mice that is less severe but also present in heterozygous animals.

**Juvenile Cln3<sup>ex7/8</sup> knock-in mice display reduced exploratory behaviour**

Having observed neurodevelopmental delays in Cln3<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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<sup>ex7/8</sup> 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,
we started measuring the latency time required by 8-week-old wild-type, heterozygous and homozygous Cln3<sup>D<sub>ex7/8</sub></sup> 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<sup>D<sub>ex7/8</sub></sup> 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<sup>D<sub>ex7/8</sub></sup> 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<sup>D<sub>ex7/8</sub></sup> mice. Cln3<sup>D<sub>ex7/8</sub></sup> 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<sup>D<sub>ex7/8</sub></sup> 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<sup>D<sub>ex7/8</sub></sup> homozygous mice and less severe motor deficits in heterozygous Cln3<sup>D<sub>ex7/8</sub></sup> mice.

**Heterozygous Cln3<sup>D<sub>ex7/8</sub></sup> mice express intermediate levels of wild-type and mutant Cln3 mRNA**

Comparative qRT-PCR was used to determine the relative expression of Cln3 mRNA transcripts in wild-type, heterozygous and homozygous Cln3<sup>D<sub>ex7/8</sub></sup> mice. Previously, it was shown that Cln3<sup>D<sub>ex7/8</sub></sup> mice express multiple variant transcripts in response to the deletion of exons 7 and 8 from the genomic sequence (Cotman et al. 2002). Because
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

Figure 2: Decreased exploratory activity in homozygous and heterozygous Cln3<sup>Δex7/8</sup> mice. Eight-week-old animals were tested in the open-field apparatus. Homozygous and heterozygous Cln3<sup>Δex7/8</sup> 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<sup>Δex7/8</sup> 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<sup>Δex7/8</sup>, n = 27; homozygous Cln3<sup>Δex7/8</sup>, n = 25).

Table 4: Behaviour of wild type, homozygous and heterozygous Cln3<sup>Δex7/8</sup> knock-in 8-month-old mice in the elevated plus maze (wild type, n = 28; heterozygous Cln3<sup>Δex7/8</sup>, n = 31; homozygous Cln3<sup>Δex7/8</sup>, n = 27) (a), forced swimming (wild type, n = 10; heterozygous Cln3<sup>Δex7/8</sup>, n = 13; homozygous Cln3<sup>Δex7/8</sup>, n = 13) (b) and visual cued water maze tests (wild type, n = 9; heterozygous Cln3<sup>Δex7/8</sup>, n = 12; homozygous Cln3<sup>Δex7/8</sup>, n = 15) (c).

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Cln3&lt;sup&gt;Δex7/8&lt;/sup&gt;</th>
<th>Cln3&lt;sup&gt;Δex7/8Δex7/8&lt;/sup&gt;</th>
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<tr>
<td>(a) Elevated plus maze:</td>
<td>14.43 ± 2.617</td>
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<td>time in open arms (%)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
</tr>
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<td>(b) Forced swimming:</td>
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<td>75.76 ± 2.364</td>
<td>65.71 ± 4.192</td>
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<td>immobility time (%)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
<td>(mean ± SEM)</td>
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<tr>
<td>(c) Visual cued water</td>
<td>71.83 ± 6.306</td>
<td>82.58 ± 6.345</td>
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<td>maze: escape latency</td>
<td>(seconds) (mean ± SEM)</td>
<td>(mean ± SEM)</td>
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<tr>
<td>time</td>
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<tr>
<td>16.20 ± 2.222</td>
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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 Cln3lacZ 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 Cln3Dex7/8, the most common JNCL mutation, may have biological function (Kitzmuller et al. 2008). Therefore, we studied the early postnatal behaviour of heterozygous and homozygous Cln3Dex7/8 knock-in mice. In

![Figure 3: Evidence for motor deficits in homozygous and heterozygous Cln3Dex7/8 mice.](image-url)

(a) When tested in the rotarod, homozygous 8-week-old Cln3Dex7/8 mice have a significantly decreased latency to fall off the rod at the speeds of 15, 20 and 24 r.p.m., while heterozygous Cln3Dex7/8 mice only exhibit decreased latency to fall at the speed of 24 r.p.m. (wild type, $n = 11$; heterozygous Cln3Dex7/8, $n = 13$; homozygous Cln3Dex7/8, $n = 17$). Mean values are plotted with confidence interval at a 95% level. Ambulatory gait was also analysed, and homozygous Cln3Dex7/8 mice have a significantly decreased displacement of (b) hind and (c) forepaws, while heterozygous Cln3Dex7/8 animals only present a decrease in (b) hind paws displacement (wild type, $n = 9$; heterozygous Cln3Dex7/8, $n = 12$; homozygous Cln3Dex7/8, $n = 15$). Mean values are plotted with SEM, $P < 0.05$. 

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homozygous Cln3^{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^{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^{ex7/8} induces a delay in the maturation of brainstem and cerebellar structures not only in homozygous but also in heterozygous Cln3^{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^{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^{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^{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 (Eliaison et al. 2007; Kovacs et al. 2006), 8-week-old homozygous Cln3^{ex7/8} mice exhibit a marked motor impairment that is evident in the rotarod test. Interestingly, Cln3^{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^{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^{ex7/8} mice and are, again, present and milder in the heterozygous Cln3^{ex7/8} mice. As the rotarod and gait analysis are highly influenced by cerebellar function, these behavioural results suggest that, as expected, homozygous Cln3^{ex7/8} mice might undergo more pronounced cerebellar degeneration. The demonstration of behaviour deficits in heterozygous Cln3^{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; Eliaison et al. 2007; Katz et al. 2008; Mitchison et al. 1999). It has been reported that Cln3^{ex7/8} knock-in mice exhibit earlier and more severe neurological disease (Cotman et al. 2002) when compared with Cln3^{lacZ} and Cln3^{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^{ex7/8} knock-in mice might be influenced by strain background effects and genetic modifiers because different strains were used (Cotman et al. 2002; Eliaison et al. 2007; Katz et al. 2008; Mitchison et al., 1999). This hypothesis is supported by the comparison of Cln3^{ex7/8} knock-in mice with the Cln3^{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^{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^{lacZ} and Cln3^{ex1-6} 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^{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^{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.

References


Early behavioural alterations in $\text{Cln}3^{\text{ex7/8}}$ mice


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