BEHAVIORAL DIFFERENCES IN A MOUSE MODEL OF BATTEN DISEASE

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ABSTRACT
Juvenile neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is an autosomal recessive human neurodegenerative disease caused by a mutation in the \textit{CLN3} gene. Symptoms of Batten disease are first observed around five years of age and include progressive visual, motor, and cognitive deterioration and seizures that lead to premature death during the third decade of life. Mouse models with differing mutations in \textit{Cln3} (the mouse homologue of \textit{CLN3}) have been used to study Batten disease. In this study, behavioral performances of \textit{Cln3} knock-in mice (KI) were compared to wild-type controls (WT) to assess cognitive or locomotor differences prior to the onset of serious Batten disease symptoms. Motor and cognitive functioning of the mice were assessed using force-plate actometers and operant chambers, respectively. There were no differences between KI and WT mice in overall locomotion or stationary movement, but KI mice engaged in more active bouts of locomotion (20 or more centimeters traveled in 2.56 seconds) than WT controls. In addition, KI mice consistently made fewer operant responses and earned fewer rewards than controls. Together, these results show that \textit{Cln3} KI mice have a distinct behavioral phenotype prior to developing debilitating Batten disease-like symptoms. Defining a behavioral profile for \textit{Cln3} KI mice may help determine the effectiveness of future treatments and therapies for this disease. Moreover, studies comparing the \textit{Cln3} KI mice to other current Batten disease models may provide insight into the diagnosis, treatment, and understanding of disease progression.

INTRODUCTION
Batten disease is a member of the neuronal ceroid lipofuscinoses (NCL), a group of autosomal recessive neurodegenerative disorders. Each of these disorders is caused by a mutation in one member of the \textit{CLN} gene family and is characterized by similar disease symptoms but a unique age of onset (Chan, Ramirez-Montalegre, and Pearce, 2009). Batten disease, known as junior neuronal ceroid lipofuscinosis (JNCL), is the most common form of NCL. Batten disease first becomes apparent around the age of five when children exhibit progressive visual deterioration leading to blindness (Weimer et al., 2009). Other symptoms include seizures, motor deficits, and cognitive decline (Weimer et al., 2009). Motor deficits associated with the disorder are characterized by cog-wheel rigidity, impaired balance, flexed posture, and abnormal shuffling gait (Hofman et al., 1999). Neuronal deterioration continues until death between 20 and 30 years of age (Weimer et al., 2009). Little is understood about the progression of Batten disease in human beings, partly because of extensive variability in disease-causing mutations resulting in a wide variety of symptoms.

Batten disease is caused by a mutation in the \textit{CLN3} gene that encodes a 438 amino acid polypeptide that likely exists as a transmembrane protein (Katz et al., 1997; Jarvela et al., 1998; Jarvela et al., 1999; Kremmidiotis et al., 1999; Margraf et al., 1999; Luiro et al., 2001; Ezaki et al., 2003; Xia and Davidson, 2003; Kyttala et al., 2004; Mao, Persaud-Sawin et al., 2004). Currently, the function of the protein product of \textit{CLN3} is not well understood; however, various studies indicate its presence in a wide variety of cellular membranes, including neuronal presynaptic vesicles and synaptosomes (Katz et al., 1997; Jarvela et al., 1998; Jarvela et al., 1999; Kremmidiotis et al., 1999; Margraf et al., 1999; Luiro et al., 2001; Ezaki et al., 2003; Xia and Davidson, 2003; Kyttala et al., 2004; Mao, Persaud-Sawin et al., 2004).
Proposed functions for CLN3 include involvement in apoptosis, proteolipid modification, endocytosis, energy metabolism, neurotransmission, and lysosomal acidification (Golabek et al., 2000; Holopainen et al., 2001; Luíro et al., 2004; Luíro et al., 2006; and Narayan et al., 2006).

Four recently developed mouse strains with various mutations in Cln3, the mouse homologue of human CLN3, have been employed as models of human Batten disease: the \( \text{Cln}3^{-/-} \) knockout (Mitchison et al., 1999), the \( \text{Cln}3^{\Delta\text{ex7/8}} \) knock-out (Katz et al., 1999), the \( \text{Cln}3^{\Delta\text{ex7/8}} \) knock-in (Cotman et al., 2002), and the \( \text{Cln}3^{\beta\text{-galactosidase}} \) reporter (Eliason et al., 2007). Studies have aimed to compare each strain both phenotypically and biochemically to the disease characteristics described in human beings. Approximately 85 percent of human Batten disease cases are caused by a 1.02kb deletion that alters the reading frame to produce a premature stop codon that excludes exons 7 and 8 (Mole, 2004). For this reason, mice with a similar deletion, as in the \( \text{Cln}3^{\Delta\text{ex7/8}} \) knock-in, are believed most closely to represent human Batten disease. Cotman et al. (2002) and Osório et al. (2009) both reported motor deficits characteristic of Batten disease in \( \text{Cln}3 \) knock-in mice compared to wild-type controls. Furthermore, heterozygous \( \text{Cln3} \) knock-in mice displayed intermediate deficits, which have also been reported in humans heterozygous for JNCL (Osório et al., 2009). Variant transcripts of both \( \text{CLN3} \) and \( \text{Cln3} \) have been observed in human beings with Batten disease and in \( \text{Cln3} \) knock-in mice, respectively (Cotman et al., 2002, Kitzmüller et al., 2008). These variants likely play an active part in producing disease-like symptoms (Kitzmüller et al., 2008). \( \text{Cln3} \) knock-in mice, however, do not display degeneration of retinal neurons that causes the trademark blindness seen as the first disease symptom in human beings (Cotman et al., 2002). The underlying basis for this observation is as yet unknown but may be related to differences in the murine and human visual systems or species-dependent differences in the expression of specific gene products. Despite this discrepancy, \( \text{Cln3} \) knock-in mice phenotypically represent a very promising model of human Batten disease.

Serious disease symptoms have been noted in \( \text{Cln3} \) knock-in mice at 10 months of age, with a 20 percent decrease in survival at one year (Cotman et al., 2002). Characterizing early behavior in these mice could help define a developmental timeline better to understand the progression of Batten disease symptoms. This information could then be used to assess the effectiveness of disease therapies and treatments and could also provide insight into the behavioral abnormalities seen in young children with Batten disease.

In this study, young \( \text{Cln3}^{\Delta\text{ex7/8}} \) knock-in and wild-type mice were compared in a variety of behavioral assays that tested both locomotor and cognitive function. Since \( \text{Cln3} \) is expressed in the mouse central nervous system during embryonic development (Eliason et al., 2007) mutations in \( \text{Cln3} \) could potentially produce detectable postnatal behavioral deficits. Batten disease is marked by cognitive and motor decline, so these two variables were examined in this study. Mice were tested using force-plate actometers to measure basic locomotor parameters such as gait, distance traveled, and exploratory behavior. Operant chambers were used to test cognitive function and the ability to learn a task by positive reinforcement. \( \text{Cln3}^{\Delta\text{ex7/8}} \) knock-in mice displayed deficits in both cognitive and locomotor function at a young age, compared to wild-type controls.

**METHODS**

Subjects: Twenty-one male mice (8 C57BL/J6 wild-type (WT) and 13 C57BL \( \text{Cln3}^{\Delta\text{ex7/8}} \) knock-in) were used in this study and were tested from postnatal day (PND) 30 to PND 150. The original knock in (KI) strain (Cotman et al. 2002) was backcrossed 16 times to WT C57BL/J6 (Jackson Laboratory, Bar Harbor, ME) and the \( \text{Cln3} \) KI animals were bred in-house. When not undergoing testing, animals were group-housed (5 animals per cage maximum) at The University of Rochester Medical Center on a 12: 12 hour light: dark cycle and were provided with food and water ad libitum. Individuals were identified via permanent ink markings on the tail prior to PND 23, and via tail tattoo thereafter. All procedures were approved by the University of Rochester Institutional Animal Care and Use Committee and conformed to FHS policy on humane care and use of laboratory animals.

During testing, mice were single-housed, allowing for controlled food deprivation to promote optimal operant task performance. Ten days prior to the start of testing, animals were single-housed with
ad libitum access to food for the first three days. Animals were weighed each day for the first three days of the 10 day pretest period. Based on the average of these weights, a target weight for each animal was determined to be 85% of this average weight. On the fourth day of the pretest period, food deprivation was begun and continued until testing was completed. Animals were allowed access to food in quantities sufficient to maintain approximately 85% of their original body weight; each animal was fed 0-4 grams of mouse chow per day depending on the current weight-target weight difference. No animal was deprived of food for more than one consecutive day and a minimum of 3g of dry food was given per weekend (Friday through Sunday).

Immediately after the last day of testing at PND 150, all animals were euthanized by exposure to CO2.

Experimental Procedure
Locomotor activity was measured using force-plate actometers (see Fowler et al., 2001). Each actometer consisted of a plexiglass box with a 280 x 280 mm² pressure-sensitive base that recorded spontaneous movement (sampling rate, 50 Hz) throughout a 25-minute testing session on PND 30, 60, 90, 120, and 150 under conditions of dim, red-light illumination. Data points were collected in real time and stored to disk on a computer for subsequent analysis. At each age except PND 30, animals were tested for one day following four days of operant testing (described below). On PND 30, animals were only tested in the actometer. Mice were weighed prior to testing, and any mouse weighing less than 45g was not used because of limits in the sensitivity of the actometer. One mouse whose target weight was <45g was fed more to surpass the 45g sensitivity cutoff, despite the fact that this was more than 85% of its original weight. Animals were always tested in the same order and each animal was assessed using the same actometer (4 different actometers were used). One of the actometers was equipped with a video camera that allowed independent confirmation of the movement sensed by the apparatus. Animals were tested at approximately the same time each day to avoid changes in activity that might be related to diurnal rhythm.

The actometer data were analyzed to characterize several movement-related variables including general locomotor activity, tremor, stereotypy, seizures, and gait. Parameters analyzed included space usage (estimated by chi-square analysis), distance of movement or total activity, number of low mobility bouts (staying within a 3cm diameter circle for 10s), number of active bouts (travelling over 20cm in 2.56s), total distance in low mobility bouts, number of rotations around the center of the chamber (the central square occupied 25% of the actometer area), percent of time spent in the center of the platform, stereotypy index (a measurement of highly repetitive, rhythmic behaviors such as sniffing and grooming, but not locomotion; stereotypy can be a sign of neurotransmitter imbalance), average total force, and variance in the total force (energy expenditure). Total activity and total distance assess basic locomotor function. By contrast, space use and the percent of time spent in the center of the chamber measure exploratory behavior. The number of low mobility bouts and activity levels during these bouts provide information about periods of rest. The variable VFz (see Table 1) is a measure of the variance in the total force and is therefore related to energy expenditure. Other variables studied but not shown in Table 1 addressed the same phenomena from slightly different perspectives.

Cognitive behavior was assessed using an operant chamber and measured by improved performance on operant tasks. Mice were placed individually into a small rectangular chamber containing a wire mesh floor, two Plexiglas walls, and two metal walls. During testing, each chamber was enclosed in a wooden box to eliminate visual distractions. One wall contained two holes, each equipped with a light sensor that, when disrupted by the mouse’s snout, revealed the mouse’s presence (See Baron and Meltzer [2001] for a detailed description of the apparatus). On the opposite wall, a chamber presented a food reward of evaporated milk. The chambers were programmed to provide a food reward when the mouse placed its snout into the right, but not the left, lit hole. At each age, each mouse was always tested in the same order using the same operant chamber (a total of six were used). Tests were performed at approximately the same time of day to minimize any potential effects of diurnal rhythm on learning the operant task. The number of times a mouse placed its snout into the correct hole,
the number of entries into the wrong hole, the number of entries into the chamber that offered food rewards, and whether or not the food reward was present at the time of entry were all recorded automatically and in real time through computer interface.

Two days prior to the first day of testing, mice were exposed to evaporated milk (in place of water) in their home cage for at least 12 hours. The day before testing, water was returned, but the mouse was deprived of food. Mice were then trained for five days to place their snouts into the correct (right) hole to receive a condensed milk reward. Each training session lasted 25 minutes. During this initial training period mice were given unlimited rewards (one reward per correct operant response). In these experiments mice were weighed immediately before and after each operant session each day (both training and testing); the posttest weights were used to estimate food restriction.

After initial training, mice were tested under a fixed interval reward program at PND 60, 90, 120, and 150. This program allowed a maximum of one reward per 60 seconds during a 25-minute session regardless of the number of correct operant responses. Mice were expected to learn to make fewer and fewer operant responses since most did not produce a reward. Mice were trained on the fixed interval reward program for five days. During this period, the time interval between correct operant responses that produce rewards was gradually increased from zero to 60 seconds. The time point extensions were 5s, 10s, 20s, 30s, and 60s. After this training, mice were tested for four consecutive days using the 60s time interval. Only data from the fourth day were used in subsequent analyses since at that point the animals had adequate time to learn the task and performance levels should therefore have been highest.

**Statistical Analysis**

Data from the force-plate actometers were analyzed using a multivariate repeated measures ANOVA to compare motor function of Cln3 KI to WT mice. Operant data were also analyzed using a repeated measures ANOVA. A two sample t-test was used to compare data between groups for each variable studied across all ages. T-tests produced no significant differences (data not shown). Significant differences (*) were values at which \( p < 0.05 \).

**Results**

The force-plate actometer data were analyzed for a variety of locomotor variables. A comprehensive comparison of these variables failed to reveal differences between WT and KI mice in the majority of locomotor parameters studied (Table 1). Significant age-specific differences were found for most of the variables studied (Table 1). These differences were expected and likely reflect changes in activity level resulting from increasing postnatal age (Serradj and Jamon, 2007). Significant differences were noted.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Between</th>
<th>Within Subjects</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Type</td>
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<tr>
<td>Space Usage</td>
<td>14.5 ± 0.58</td>
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<tr>
<td>Total Activity</td>
<td>106144 ± 2156.86</td>
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<td>Low Mobility Bouts</td>
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<td>Activity During Low Mobility Bouts</td>
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<tr>
<td>% Time in Center</td>
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<tr>
<td>VFz</td>
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<td>0.110</td>
</tr>
<tr>
<td>Locomotor</td>
<td>39165 ± 914.15</td>
<td>0.192</td>
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Table 1. Statistical analysis of force-plate actometer data from Cln3 KI (n=13) and WT (n=8) mice. Repeated measures ANOVAs were performed for each of the variables shown. P values for a few select variables are shown; values are bold for \( p < 0.05 \). All variables not shown did not exhibit significant differences between WT and KI mice in the number of active bouts; however, differences were only significant on PND 150 (Figure 1).

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Figure 1. Number of active bouts of locomotion in knock-in (KI, n=13) and WT (Control, n=8) mice at five postnatal ages (in days). An active bout of locomotion was defined as movement over 20cm in 2.56s. A significant difference between groups was seen on PND 150. \( *p < 0.05 \).

Analysis of the operant data revealed that Cln3 KI mice made fewer operant responses and earned fewer rewards (Figure 2) than WT controls, suggesting that both cognitive function and learning were impaired in the Cln3 KI mice. However, these differences were significant only on PND 90 and 120. The duration of operant responses also differed between groups with KI mice making longer operant

Figure 2. Number of operant responses made (a) and rewards earned (b) by knock-in (KI, n=13) and wild WT (Control, n=8) mice at four ages. The number of operant responses was significantly greater in WT...
compared to KI mice on PND 90 and PND 120. The number of rewards earned was significantly greater WT compared to KI mice on PND 90 and PND 120. *p < 0.05.

responses than controls (Figure 3a). This difference was significant on PND 60 and 150. Reward reaction time, defined as the time it took an animal to retrieve a reward after making an operant response was also used to assess motor function. No differences were found between groups for this variable (Figure 3b).

Figure 3. Duration of operant responses (a) and reward reaction time, RT, (b) at four ages in knock-in (KI, n=13) and WT (Control, n=8) mice. (a) WT mice exhibited significantly shorter responses compared to KI mice on PND 60 and PND 150. (b) Reward reaction time was defined as the time between an operant response and retrieval of reward (travel time to reward). No significant differences were found between groups. *p < 0.05.

DISCUSSION

Cln3 KI mice displayed deficits in cognitive performance and one aspect of motor function from PND 30 to PND 150. Deficits at these early ages are an important addition to our understanding of Batten disease symptom progression. Serious symptoms have been reported in Cln3 KI mice at 10 months (~PND 300), with a 20% decrease in survival at 12 months (PND 360) (Cotman et al., 2002). This study found differences in behavior at much younger ages, suggesting that milder disease symptoms can be detected by PND 30. A previous study by Osório et al. (2009) found differences in neonatal neurological reflexes from PND 3 to PND 21 and motor differences at 8 weeks (PND 60), supporting the assumption that behavioral differences exist in these mice prior to the later onset of more visible and serious disease symptoms. This is the first study to examine learning in Cln3 KI mice, even though cognitive decline is a common symptom associated with Batten disease in human beings.

The locomotor data revealed very few differences between KI mice and WT controls, but this is not surprising, given the young ages of the mice. These data confirm a previous study by Osório et al. (2009) that detected no differences between Cln3 KI and WT mice in basic locomotor activity at 8 weeks of age, including total distance traveled. However, this previous study found that 8-week-old Cln3 KI mice spent significantly less time in the center of a platform compared to controls, whereas the current study found no difference in this variable (p=0.059, Table 1). Animals in the present study were tested at five different ages, whereas Osório et al. (2009) tested the animals only once—at 8 weeks—and used a different method of data collection. The discrepancy between the two studies may be caused by differences in methodology or may arise from the small sample sizes in each study. Interestingly, Osório et al. (2009) found no evidence of anxious behavior when animals were tested in the elevated plus maze, suggesting that the difference in time spent in the center of the platform resulted from differences in
exploratory activity rather than anxiety. To clarify the discrepancies between the two studies, further experiments with larger sample sizes are needed. Including additional ages in future studies may also help to pinpoint differences in exploratory behavior between Cln3 KI and WT mice.

One significant difference in locomotor behavior between Cln3 KI and WT mice was found: KI mice displayed a greater number of active bouts of locomotion (moving over 20cm in less than 2.56 seconds) than WT controls (Figure 1). However, a significant difference between the groups was noted only at PND150 (Figure 1). The reason for this difference in activity is unclear, although the increased activity level in the Cln3 KI may be related to progressive neurodegeneration at this later age. Additional studies of a wider range of ages may be required to resolve this question.

Cognitive behavior and learning were significantly decreased in Cln3 KI mice compared to controls. The KI mice made fewer operant responses and earned fewer rewards; these differences were significant at PND 90 and PND 120 (Figure 2). This finding was unexpected, given the early onset age and the fact that the difference was no longer seen at PND 150 when Batten disease is marked by progressive degeneration. Further examination revealed that the maximum number of rewards earned by control mice already had peaked between PND 60 and PND 120 (Figure 2b). The controls began to make fewer operant responses at the last age studied (Figure 2a), but this may result from the mouse learning that not every response was rewarded. By contrast, the number of operant responses and rewards earned by the KI mice continued to increase steadily at later ages (Figure 2). In fact, the rate of increase in rewards earned in successively older animals (the slope of the lines in Figure 2b) for PND 60 to PND 120 was similar for control and KI mice, although the KI values started at a lower level. This implies that the differences in performance between control and KI mice are attributable to different baseline learning levels rather than different learning rates as a result of training.

The KI mice also displayed different operant response durations, taking significantly longer to respond than WT controls (Figure 3a). The response duration increased in KI mice from PND 90 to PND 150, whereas it stayed constant for the controls during the same period. It was expected that the number of longer responses would stay the same or decrease as learning occurred because longer responses were not specifically rewarded whereas other kinds of behavior (for example, making more operant responses) were. Longer response times in KI mice may arise from a deficit in task learning, but a deficit in motor function could also have increased the duration of operant responses. To assess this, we measured reward reaction time, defined as the amount of time each animal took to make an operant response and then retrieve the food reward. When assessed in this way, no differences were found in reward reaction time between Cln3 KI and control mice (Figure 3b), complementing the force-plate actometer findings, and supporting the more general conclusion that there were no differences in overall activity between the two groups of mice. This finding also implies that the difference in operant response durations was not caused by motor deficits in the KI mice.

Considering the findings of Osório et al. (2009) and the current study, a behavioral timeline of symptom progression in Cln3 KI mice can be constructed (Figure 4) that can be used to assess the onset of disease symptoms and the rate of disease progression, particularly with regard to neurodegeneration at early ages. In addition, this timeline may be used to compare data from studies using other mouse models and documented cases of Batten disease in human beings. Because the exon7/8 deletion employed in the Cln3 KI mouse used in these studies is thought to represent the most common mutation seen in Batten disease, any discrepancies in disease symptoms or progression may provide further insight into understanding the genetics and protein expression of the disease. Defining when neuronal degeneration begins would be particularly valuable since developing therapies effective prior to extensive neuronal death will be critical to limiting or even reversing disease progression.

Behavioral assessment is critical for a full understanding of diseases such as Batten disease. While biochemical analysis can help delineate the molecular causes of disease symptoms and genetic analysis can clarify hereditary components, behavioral observations are crucial for monitoring the overt symptoms of the disease and their severity, particularly when assessing the effectiveness of disease treatment. Studies that combine molecular, genetic, and biochemical approaches with behavioral analysis are therefore more effective for understanding and treating the disease. Moreover, current studies
utilizing such a multipronged approach are much more likely to generate new ideas and provide groundwork for future molecular and physiological studies.

Figure 4. Preliminary behavioral timeline describing motor and cognitive deficits reported in Cln3 KI mice from birth to one year of age. The timeline combines data from the three studies shown (unmarked box represents data from the current study). Scale is relative (shown by ticks).

In summary, the main findings of the current study and of Osório et al. (2009) revealed behavioral differences in Cln3 KI mice prior to the onset of serious disease symptoms. Based on these studies, a preliminary timeline of disease progression in Cln3 KI mice was constructed for better understanding disease progression, for comparing the Cln3 KI mouse with other models of Batten disease, and for monitoring the effectiveness of treatments or therapies as they develop. The behavioral data from this study should help in assessing the behavior of very young (1-4 year-old) children with Batten disease as earlier treatments are explored to limit or prevent devastating later symptoms of the disease.

LITERATURE CITED


ACKNOWLEDGEMENTS
This project was supported by NIH N540580 and NIEHS ES01247 and was made possible by the University of Rochester Summer Scholars Program. Thanks to URMC staff for advice and use of facilities; to Kristina Long, Adam Katz, Rebecca Fallon, Christina Caraballo, Aynsley Duncan, and Colin Gordon for data collection; to Troy Zarcone, Dena Carbonari, Timothy Curran, and David Pearce for guidance and mentoring; to Professor Steve Klug for his support through TCNJ’s Biology Internship Program; and to Professor Jeffery Erickson for editorial help.