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ORIGINAL ARTICLE

Neurological Dysfunction in Early Maturity of a Model for Niemann–Pick C1 Carrier Status

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Abstract Autosomal recessive inheritance of *NPC1* with loss-of-function mutations underlies Niemann–Pick disease, type C1 (NP-C1), a lysosomal storage disorder with progressive neurodegeneration. It is uncertain from limited biochemical studies and patient case reports whether *NPC1* haploinsufficiency can cause a partial NP-C1 phenotype in carriers. In the present study, we examined this possibility in heterozygotes of a natural loss-of-function mutant *Npc1* mouse model. We found partial motor dysfunction and increased anxiety-like behavior in $Npc1^{+/-}$ mice by 9 weeks of age. Relative to $Npc1^{+/+}$ mice, $Npc1^{+/-}$ mice failed to show neurodevelopmental improvements in motor coordination and balance on an accelerating Rotarod. In the open-field test, $Npc1^{+/-}$ mice showed an intermediate phenotype in spontaneous locomotor activity compared with $Npc1^{+/+}$ and $Npc1^{-/-}$ mice, as well as decreased center tendency. Together with increased stride length under anxiogenic conditions on the DigiGait treadmill, these findings are consistent with heightened anxiety. Our findings indicate that pathogenic *NPC1* allele carriers, who represent about 0.66 % of humans, could be vulnerable to motor and anxiety disorders.

Keywords NPC1 · Niemann–Pick disease type C · haploinsufficiency · Carrier · Heterozygote disease · Neurological

Introduction

Niemann–Pick disease, type C1 (NP-C1; MIM 257220) is an autosomal recessive lysosomal storage disorder, caused by loss-of-function mutations in *NPC1*. NP-C1 cells have a distinct biochemical phenotype of lysosomal cholesterol and glycosphingolipids overload, which subsequently causes cellular damage and dysfunction. Clinically, visceral symptoms, such as hepatosplenomegaly, are most prominent in patients

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with NP-C1 presenting at an age younger than 6 years [1]. For patients aged ≥6 years, the main clinical presentations are neurological and psychiatric [1], which include cerebellar ataxia, loss of motor coordination, dysarthria, dysphagia, vertical supranuclear gaze palsy, seizures, psychosis, and dementia [1, 2]. The neurological and psychiatric symptoms are related to early loss of cerebellar Purkinje cells, later loss of hippocampal and cortical neurons, and impaired myelination [3–7]. Disease onset can occur at any age, with a predominant childhood manifestation that leads to premature death before adulthood [8]. The only approved drug treatment is with miglustat, a glucosylceramide synthase inhibitor. For patients with NP-C1 who were treated with miglustat continuously for an average of 2 years, the majority were categorized as improved/stable in their neurological disease progression [9]. Currently, *NPC1* carriers are generally considered to be clinically unaffected, but it is uncertain whether *NPC1* haploinsufficiency can predispose *NPC1* carriers to moderate and potentially subclinical NP-C1 phenotypes.

The most recent estimate of the carrier frequency for pathogenic *NPC1* alleles is 0.66 % [10]. Biochemically, *NPC1* carriers have an intermediate phenotype in lipid regulation and metabolism. Cultured human and feline *NPC1* obligate heterozygote skin fibroblast cells demonstrated an intermediate rate of cholesterol esterification, production of cholesteryl esters, and unesterified cholesterol storage level compared with healthy and homozygote *NPC1* cells [11, 12]. Human fibroblasts also expressed an *NPC1* dosage-dependent change in ABCA1 protein expression and apolipoprotein A-I-mediated lipid efflux [13]. In human *NPC1* carriers, the concentrations of plasma oxysterols, 7-ketocholesterol, and cholestane-3β-5α-6β-triol are commensurately elevated between healthy and NP-C1 levels [14]. The liver in heterozygous *Npc1*$^{+/-}$ mice compared with homozygous *Npc1*$^{+/+}$ and presymptomatic *Npc1*$^{-/-}$ mice at 35 days of age has an intermediate level of sterol regulatory element-binding transcription factor 1 and 2 protein expression [15]. Subsequent mouse model studies revealed that a high-fat diet promoted greater weight gain, and impaired glucose metabolism and insulin resistance in *Npc1*$^{+/-}$ mice [16, 17].

Despite harboring an intermediate biochemical phenotype, *NPC1* carriers are generally considered subclinical. *NPC1* haploinsufficiency has been found to be associated with early-onset and morbid adult obesity in a recent genome-wide association study of European populations [18], but little is known of other health risks. Two studies that report central nervous system abnormalities in NP-C1 animal models suggest potential neurological impairment in aging *NPC1* carriers. Ten-year-old *NPC1* heterozygous cats have ectopic dendrites in cortical pyramidal neurons, with increased GM2 ganglioside [12]. *Npc1*$^{+/-}$ mice examined at advanced age (2 years) show neurodegeneration with a significant loss of Purkinje neurons, and increases in brain cholesterol and hyperphosphorylated tau [19]. Consistent with these animal studies, there are also some case reports of tremor, parkinsonism, and increased foamy storage cell in bone marrow manifesting in aging *NPC1* carriers [20–22]. Also, an international genetic screen for the prevalence of NP-C1 in adults with neurological and psychiatric disorders revealed an apparent enrichment of *NPC1* carriers within this patient population [23]. However, it is unclear in these cases whether pathogenic *NPC1* allele carrier status alone can cause neurological and psychiatric symptoms, or whether a neuropsychiatric phenotype is the consequence of a vulnerability induced by *NPC1* allele carrier status that emerges only after interaction with other genetic defects and/or environmental factors. Furthermore, the timing of the onset of the neuropsychiatric phenotype is not understood.

In this study, we interrogated the motor and behavioral phenotypes associated with *NPC1* haploinsufficiency to early neurodevelopmental maturity, using the BALB/cJ *Npc1*^{nih} mouse model [24]. The use of a mouse model allows us to control genetic background and environment, examining the specific impact of *NPC1* haploinsufficiency, which would not be feasible with human subjects. Our study revealed that *Npc1*$^{+/-}$ mice have partially impaired locomotor activity and increased anxiety-like behavior in early maturity, intermediate to the phenotypes of *Npc1*$^{-/-}$ mice. Therefore, pathogenic *NPC1* carriers could be at risk of attenuated NP-C1 disease.

Methods

Animals

Breeding pairs of heterozygous BALB/cJ *Npc1*^{nih} (*Npc1*$^{+/-}$) mice were obtained as a kind gift from Dr. Steven Walkley (Albert Einstein College of Medicine, NY, USA) [24]. These mice were maintained at the Florey Core Animal Services Facility, on Barastoc standard rodent feed (Ridley Corporation, Victoria, Australia). Food and water were available *ad libitum*.

Timed mating of 14 female and 11 male *Npc1*$^{+/-}$ mice produced *Npc1*$^{+/+}$ ($n_{female}=11$; $n_{male}=7$), *Npc1*$^{+/-}$ ($n_{female}=15$; $n_{male}=15$), and *Npc1*$^{-/-}$ ($n_{female}=9$; $n_{male}=5$) littermates used in this study. We studied all the mice that were generated to a maximum number of 15 per subgroup; as a result, only the *Npc1*$^{+/-}$ subgroup was sex matched. Mouse genotypes were identified using real-time polymerase chain reaction with probes designed for *Npc1* (Transnetyx, Cordova, TN, USA). After weaning at postnatal day (P) 21, male and female mice were housed separately, with a maximum of 6 mice of mixed genotype per cage, and maintained on a 12-h light–dark cycle. The mice were monitored daily, and from P24, weighed every 2–3 days, then daily on weekdays from P36 until the end of the experiment at P71.

Experimental procedures were approved by the Florey Animal Ethics Committee and were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, Eighth Edition (2013). The experimenter was blinded to the genotype of experimental animals, which was decoded at the completion of behavioral tests.

Behavioral Tests

Mice were subjected to motor coordination and balance tests at the age of 5, 7, and 9 weeks. The tests were performed separately on consecutively days at the respective age.

Rotarod Test

Balance and motor coordination of mice were assessed using an accelerating Rotarod (PanLab, Barcelona, Spain). The electronically controlled rotating rod (30 mm diameter) accelerates at a constant rate from 4–40 rpm over a 5-min period. When a mouse falls off the rotating rod onto the plastic platform below, the latency to fall (seconds) and the rotation speed (rpm) were recorded. The total distance travelled was calculated from these measures. The experiment consists of a training session and a testing session over 2 consecutive days. Each session consists of 3 trials that were at least 1 h apart. The average of 3 trials was used in the final analysis.

Open-field Test

Spontaneous locomotor and exploratory activities of mice were assessed in an open-field test. Each mouse was placed in the center of a Plexiglass locomotor cell (27.3 cm × 27.3 cm × 20.3 cm) connected to three 16-beam infrared arrays on the *X*, *Y*, and *Z*-axes (Med Associates Inc., St. Albans, VT, USA). Mouse spontaneous locomotor activity over 15 min was measured in 5 min time bins by a computer and analyzed using Activity Monitor v.6.02 (Med Associates Inc.). Locomotor activities measured include distance travelled, ambulatory counts, vertical counts, jump counts, and average velocity. For anxiety-like behavior assessment, the center of the open-field arena (15 cm × 15 cm) was defined with start and end (*x*, *y*) coordinates as (4, 4) and (12.5, 12.5), respectively.

DigiGait Analysis

Gait was assessed using a DigiGait Imaging System (Mouse Specifics Inc., Boston, MA, USA), which consists of a transparent treadmill belt encased within a Perspex chamber. To improve contrast for automated analysis, the paws of the mice were colored with red food dye. Mice were placed in the chamber, and the treadmill belt was accelerated rapidly to 20 cm/s. As the mouse walked on the motorized transparent treadmill, its gait was captured by a high-speed digital camera placed underneath the transparent treadmill. The footprints from a segment of 3–5 s of video were then analyzed using DigiGait v. 9.9 (Mouse Specifics Inc.), which quantified multiple gait indices, including stride length, stride time, swing time, braking time, stance width, stride frequency, gait symmetry, and ataxia coefficient. The treadmill belt and Perspex chamber were cleaned with 80 % (v/v) ethanol between each animal testing. As the $Npc1^{-/-}$ mice were incapable of performing on the DigiGait at 9 weeks, owing to severe ataxia, this genotype was removed from the final analysis. The $Npc1^{+/+}$ and $Npc1^{+/-}$ mice that failed to walk on DigiGait were also removed from final analysis.

Statistical Analysis

Mouse behavioral data analyses were performed using Stata v.13 IC (Stata Corp, College Station, TX, USA). Data were transformed as indicated to ensure satisfaction of distributional assumptions for statistical testing. Owing to the repeated-measure nature of the data, and based on the data distribution, data were analyzed using random effects generalized least squared regression or random effects negative binomial regression, with the animals as a random effect, or median regression with each animal as an individual cluster. All analyses were adjusted for age and sex, and for Rotarod analysis, data were also adjusted for training/testing day. The analyses also included examination of the interaction between genotype and age.

Results

Body Weight

Fed a standard diet, there was no body weight difference between mice of all genotypes up to P28 (Fig. S1). We observed from P29 onwards that male mice became significantly heavier. Over the course of the present study (71 days), the age-dependent gain in body weight was similar between sex-matched $Npc1^{+/+}$ and $Npc1^{+/-}$ littermates, consistent with previous findings [25, 26]. Compared with $Npc1^{+/+}$ and $Npc1^{+/-}$ littermates, the body weight of $Npc1^{-/-}$ mice decreased dramatically, as expected [14, 27], from 7 weeks of age for males and from 8 weeks of age for females.

Npc1$^{+/-}$ Mice Express Impaired Age-dependent Motor Coordination

To assess the effect of *Npc1* haploinsufficiency on motor coordination and balance, we tested $Npc1^{+/+}$, $Npc1^{+/-}$, and $Npc1^{-/-}$ mice on an accelerating Rotarod at 5, 7, and 9 weeks of age. The data were analyzed as distance travelled on the

accelerating Rotarod, which took into account the rotation speed and latency to fall (Fig. 1; Fig. S2 and Table S1). There was no significant difference in the distance travelled on the Rotarod by mice of all genotypes between training and testing days ($p=0.66$). There was a significant interaction of genotype with age ($p<0.0001$), with the main significant effect between *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice, but not *Npc1*$^{+/+}$ and *Np1*$^{+/-}$ mice, over age. As expected, the distance travelled by *Npc1*$^{-/-}$ mice was significantly less than *Npc1*$^{+/+}$ mice (cube root transformed, $b=-2.9$; $p<0.0001$), and markedly decreased progressively with age (Fig. 1). The distance travelled by *Npc1*$^{+/-}$ mice on the Rotarod did not differ significantly from that of *Npc1*$^{+/+}$ mice (cube root transformed, $b=0.1$; $p=0.7$). However, examining the change in distance travelled at 7 and 9 weeks of age compared with 5 weeks of age, *Npc1*$^{+/+}$ mice showed improved motor coordination and balance with maturity (to 9 weeks), while *Npc1*$^{+/-}$ mice failed to mature in these domains (Fig. 1). Male *Npc1*$^{+/-}$ and *Npc1*$^{-/-}$ mice were significantly less able to maintain their balance and coordination on the Rotarod compared with female *Npc1*$^{+/-}$ and *Npc1*$^{-/-}$ mice, but there was no sex difference in the Rotarod performance of *Npc1*$^{+/+}$ mice (Table S1).

Spontaneous Locomotion Impairment in *Npc1*$^{+/-}$ Mice

To appraise whether *Npc1* haploinsufficiency affects spontaneous locomotor activity and novel environment exploratory

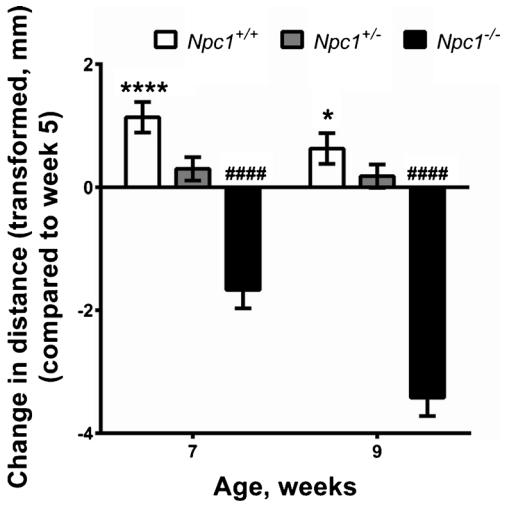

Fig. 1 *Npc1*$^{+/-}$ mice express impaired age-dependent motor coordination. The change in distance travelled on an accelerating Rotarod by *Npc1*$^{+/+}$ ($n_{female}=11$; $n_{male}=7$), *Npc1*$^{+/-}$ ($n_{female}=15$; $n_{male}=15$) and *Npc1*$^{-/-}$ ($n_{female}=9$; $n_{male}=5$) at 7 and 9 weeks of age compared with 5 weeks of age revealed impaired motor development. Statistical analysis: cube root transformation was applied to ensure satisfaction of distributional assumptions prior to analysis by random effects generalized least squares regression with the animals as a random effect. All analyses were adjusted for age, sex, and training/test day. Data represent change in cube root of distance travelled (mm) ± SE compared with week 5. *$p<0.05$, ****$p<0.0001$ (*Npc1*$^{+/+}$); ####$p<0.0001$ (*Npc1*$^{-/-}$)

behavior, we subjected *Npc1*$^{+/+}$, *Npc1*$^{+/-}$, and *Npc1*$^{-/-}$ mice to an open-field test at 5, 7, and 9 weeks of age and analyzed their horizontal and vertical movements for 15 min. Horizontal movement indices in the total arena analyzed were total ambulatory distance travelled, total ambulatory count, and average velocity. Compared with *Npc1*$^{+/+}$ mice, the total ambulatory distance travelled in the entire arena by the *Npc1*$^{+/-}$ mice was not different, whereas that of *Npc1*$^{-/-}$ mice was significantly decreased by 737.6 ± 112.6 cm (Fig. 2A; Fig. S3A). There was a significant interaction of genotype with age ($p=0.01$), with the main significant effect detected between *Npc1*$^{+/+}$ and *Np1*$^{-/-}$ mice over age. By 9 weeks of age, the total ambulatory distance travelled by *Npc1*$^{+/+}$ mice increased significantly by 183.2 ± 93.5 cm compared with 5 weeks of age ($p=0.05$), whereas there was no significant change detected in *Npc1*$^{+/-}$ and *Np1*$^{-/-}$ mice. The average velocity of *Npc1*$^{+/-}$ mice movement was significantly slower than *Npc1*$^{+/+}$ mice, and intermediate between *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice (Fig. 2B; Fig. S3B).

Vertical movements such as rearing and jumping are normal exploratory behavior of rodents in novel environments [28]. The incidence rate ratio (IRR) of jump counts showed that the expected jump counts in the total arena of both *Npc1*$^{+/-}$ and *Npc1*$^{-/-}$ mice were significantly less than *Npc1*$^{+/+}$ mice (Fig. 2C; Fig. S4A). *Npc1*$^{+/-}$ and *Npc1*$^{-/-}$ mice jumped with only about 30 % and about 10 %, respectively, of the frequency of *Npc1*$^{+/+}$ mice. Overall, male mice jumped with only about 40 % of the frequency of female mice. Further comparison of male mice with female mice within each genotype group showed that *Npc1*$^{+/+}$ and *Npc1*$^{+/-}$ male mice jumped with only about 10 % and 40 %, respectively, of the frequency of their female counterparts. There was no significant difference in jump count IRR between male and female *Npc1*$^{-/-}$ mice, which could be attributed to maximal impairment in both sexes.

Consistent with decreased jump count IRR, vertical count IRR also revealed significantly reduced vertical rearing activity in *Npc1*$^{+/-}$ and *Npc1*$^{-/-}$ mice (Fig. 2D; Fig. S4B). The IRRs for vertical rearing counts of *Npc1*$^{+/-}$ and *Npc1*$^{-/-}$ mice were only about 40 % and 10 %, respectively, that of *Npc1*$^{+/+}$ mice. There was a significant interaction of genotype with age for vertical count IRR ($p=0.03$), with the main significant interaction between *Npc1*$^{+/+}$ and *Npc1*$^{+/-}$ mice over age. Compared with their vertical rearing counts at 5 weeks of age, the *Npc1*$^{+/+}$ mice had significantly increased IRRs, by about 170 % and 190 %, at 7 and 9 weeks of age, respectively, but for the *Npc1*$^{+/-}$ mice there was no developmental increase in vertical rearing performance at 7 weeks of age, and their performance took until 9 weeks of age to exhibit a significant improvement (about 180 %). As expected, there was no significant improvement in the vertical rearing count over age for *Npc1*$^{-/-}$ mice, owing to their inherent motor impairment.

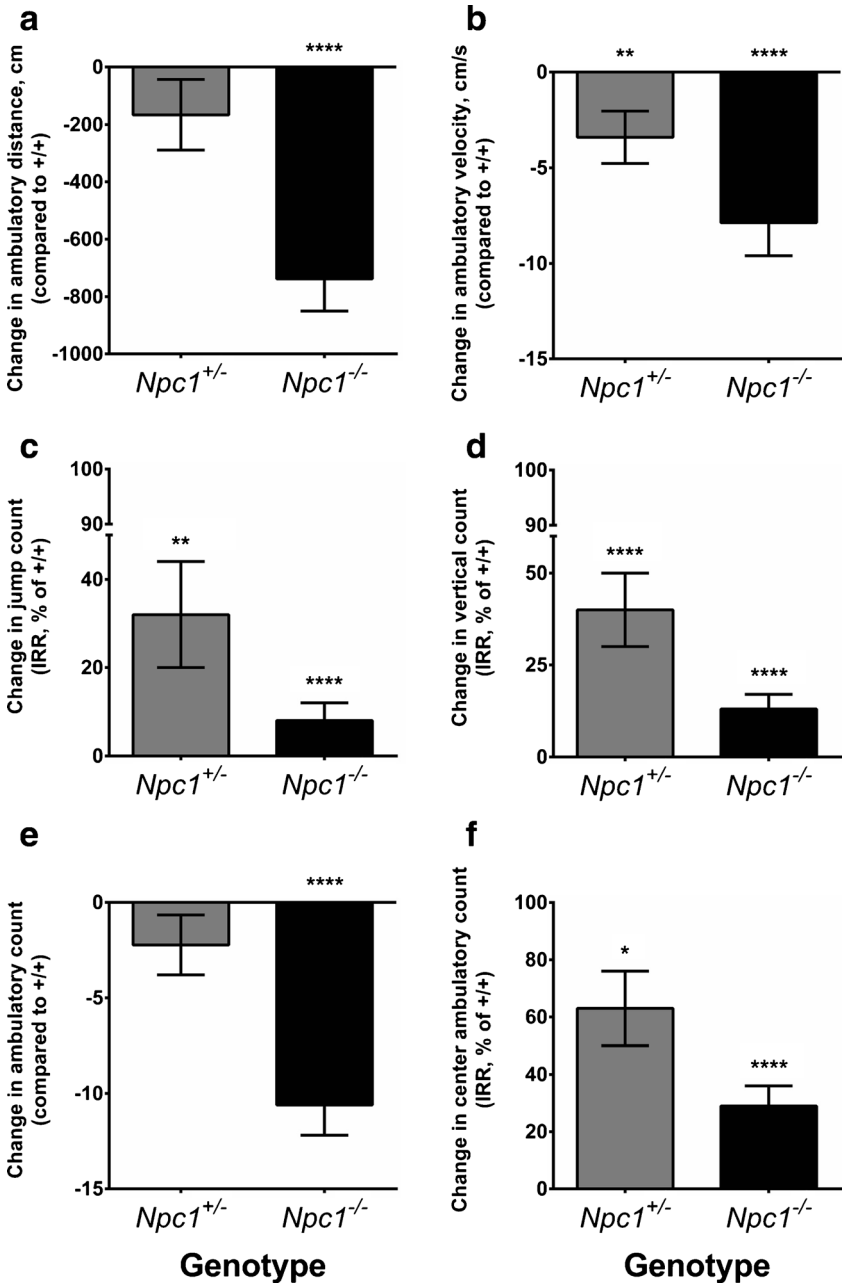

Fig. 2 Spontaneous locomotion impairment and increased anxiety-like behavior in *Npc1*$^{+/-}$ mice. *Npc1*$^{+/-}$ mice ($n_{female} = 15$; $n_{male} = 15$) express intermediate spontaneous locomotor activity indices compared with normal *Npc1*$^{+/+}$ ($n_{female} = 11$; $n_{male} = 7$) and homozygous *Npc1*$^{-/-}$ ($n_{female} = 9$; $n_{male} = 5$) mice in the open-field test performed at 5, 7, and 9 weeks of age. Data represent change ± SE compared with the *Npc1*$^{+/+}$ reference (0 for **A**, **B**, **E**, 100 % for **C**, **D**, **F**). **A** Differences in ambulatory distance travelled; (**B**) differences in average velocity; (**C**) differences in jump count incidence rate ratio (IRR); (**D**) differences in vertical count IRR; and (**E**) differences in ambulatory count (square root transformed). **F** Significantly reduced IRR for center ambulatory count in the *Npc1*$^{+/-}$ mice compared with *Npc1*$^{+/+}$ mice (100 % reference) indicates increased anxiety. Statistical analysis: (**A**, **B**, **E**) random effects generalized least squares regression or (**C**, **D**, **F**) random effects negative binomial regression with the animals as a random effect. All analyses were adjusted for age and sex. There was a significant interaction effect between the genotypes and age for ambulatory distance travelled ($p = 0.01$), and vertical count ($p = 0.03$). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ (*Npc1*$^{+/+}$)

Increased Anxiety in *Npc1*$^{+/-}$ Mice

In addition to allowing us to examine unforced and voluntary locomotion, the open-field test is classically used to assess anxiety-like behavior in rodents [29]. Mice typically spend a greater amount of time exploring the periphery of an open-field arena than the unprotected center area; with the avoidance of the center area interpreted as anxiety. We found that *Npc1*$^{-/-}$ mice exhibited significantly decreased ambulatory count in the total arena and center ambulatory count IRRs compared with *Npc1*$^{+/+}$ mice, consistent with motor impairment (Fig. 2E, F; Fig. S5). The ambulatory count in the total arena of the *Npc1*$^{+/-}$ mice did not differ significantly from that of the *Npc1*$^{+/+}$ mice (Fig. 2E). However, the ambulatory count IRR of *Npc1*$^{+/-}$ mice in the center of the arena was only about 60 % that of *Npc1*$^{+/+}$ mice (Fig. 2F), indicative of increased anxiety. Male mice entered the center of the arena at about 60 % of the incidence rate of female mice.

Npc1$^{+/-}$ Mice have Altered Stride

To appraise gait parameters sensitively, we examined the mice with the DigiGait motorized treadmill system [30–32]. This is a forced locomotor test, which can be used to highlight gait abnormalities not detectable in a voluntary locomotor test. *Npc1*$^{+/-}$ mice were evaluated on the DigiGait treadmill

walking at a constant speed of 20 cm/s. Owing to severe motor impairment, the *Npc1*$^{-/-}$ mice failed to perform on the DigiGait and were excluded from final data analysis for this experiment. A stride is defined as a complete step cycle composed of stance and swing phases involving all 4 paws. *Npc1*$^{+/-}$ mice expressed significantly altered stride measures compared with *Npc1*$^{+/+}$ mice. The stride length and duration of *Npc1*$^{+/-}$ mice were both significantly longer than *Npc1*$^{+/+}$ mice, with a concomitant decrease in stride frequency (Table 1; Figs S6–S9). Male mice had greater stride length, longer stride duration, and reduced stride frequency compared with their female counterparts, which was exaggerated in male *Npc1*$^{+/-}$ mice (Figs S6–S8).

The ataxia coefficient is an index of step-to-step variability. This index was significantly decreased in the forelimbs of *Npc1*$^{+/-}$ mice, indicating an improved consistency in the gait changes observed, compared with *Npc1*$^{+/+}$ mice (Table 1; Fig. S9). However, at 9 weeks of age, there was a significant increase in *Npc1*$^{+/-}$ mice forelimbs ataxia coefficient compared with 5 weeks of age, while there was no change in *Npc1*$^{+/+}$ mice forelimb ataxia coefficient with age.

There was no significant difference in the stance and swing durations between *Npc1*$^{+/+}$ and *Npc1*$^{+/-}$ mice (Table S2). The stance width, an indication of postural adjustment for stability, was similar between *Npc1*$^{+/+}$ and *Npc1*$^{+/-}$ mice. Gait symmetry was similar between both genotypes.

Discussion

To our knowledge, these are the first data to reveal motor and behavioral deficits in early maturity of *Npc1*$^{+/-}$ mice. Previous studies have reported that *NPC1* haploinsufficiency results in an intermediate biochemical deficit in obligate heterozygous human plasma samples, human and feline skin fibroblast cells, and mouse liver [11–15], cortical pyramidal neuron abnormalities in heterozygous *NPC1* cats [12], and neurodegeneration in aged *Npc1*$^{+/-}$ mice [19]. These findings suggest *NPC1* carriers could be predisposed to moderate and potentially subclinical neurological impairments, and may be the basis of motor syndromes reported in limited studies of *NPC1* carriers [20, 21]. The motor disturbance in these few case studies could not be attributed definitively to these individuals' *NPC1* heterozygosity, as humans are inherently heterogeneous with respect to their genetic background and environments. By studying an *Npc1* mouse model under controlled laboratory conditions, we demonstrated that partial motor dysfunction and increased anxiety-like behavior are, indeed, attributable to *Npc1* haploinsufficiency in young mice up to 9 weeks of age. Therefore, subclinical *NPC1* carriers may represent a previously unrecognized disease burden, deserving more consideration in research and medical practice.

Cerebellar ataxia is a prominent feature of NP-C1. As previously reported [27], our *Npc1*$^{-/-}$ mice demonstrated severely impaired motor coordination and balance, which deteriorates with age, as determined on an accelerating Rotarod and in an open-field test, to a degree of severity that precluded DigiGait analysis. By comparison, *Npc1*$^{+/-}$ mice showed comparable motor coordination and balance to that of *Npc1*$^{+/+}$ mice on the Rotarod. However, while *Npc1*$^{+/+}$ mice improved motor coordination and balance with maturity (to 9 weeks), *Npc1*$^{+/-}$ mice failed to mature in these domains. Therefore, *Npc1*$^{+/-}$ mice have impaired motor development.

Evaluation of voluntary movements using an open-field test showed that *Npc1*$^{+/-}$ mice have a partial loss of both voluntary horizontal and vertical movements. In the horizontal movement, *Npc1*$^{+/-}$ mice showed an intermediate decrease in average velocity compared with *Npc1*$^{+/+}$. Despite their lower average velocity, the ambulatory count and ambulatory distance travelled by the *Npc1*$^{+/-}$ mice in the total arena was not different to *Npc1*$^{+/+}$ mice. Taken together, these results suggest that the voluntary horizontal movement of *Npc1*$^{+/-}$ mice is slower and continuous, whereas *Npc1*$^{+/+}$ mice move quickly and intermittently. The *Npc1*$^{+/-}$ mice also exhibited an intermediate phenotype in their voluntary vertical movement. The IRR indicated that *Npc1*$^{+/-}$ mice are less likely to jump or make vertical rearing movements than *Npc1*$^{+/+}$ mice, but are not as severely disabled as *Npc1*$^{-/-}$ mice. Loss of vertical movement can indicate postural and gait abnormalities [33]. These differences were not immediately discernible in the *Npc1*$^{+/-}$ mice by visual inspection, but were detected using a sensitive computer-assisted locomotor array in the open-field

Table 1 DigiGait indices with significant difference between *Npc1*$^{+/+}$ (n_{female} = 10; n_{male} = 5–6) and *Npc1*$^{+/-}$ (n_{female} = 14; n_{male} = 14–15) mice, measured at 5, 7, and 9 weeks of age

	Change in *Npc1*$^{+/-}$ (compared with *Npc1*$^{+/+}$)	SE	95 % CI	*p*-Value
Stride length (cm)	0.20	0.100	0.01–0.30	0.04
Stride duration (ms)	0.01	0.004	0.0004–0.0200	0.04
Stride frequency (strides/ms)	–0.11	0.050	–0.22 to –0.01	0.04
Ataxia coefficient (forelimbs)	–0.13	0.070	–0.260 to –0.004	0.04

Random effects generalized least squares regression with animals as a random effect, adjusted for age and sex

CI Confidence interval

test. Therefore, *NPC1* carriers, who are generally regarded as clinically unaffected, may be predisposed to subtle locomotor impairments that may become apparent as neurodevelopmental delay, and as yet have not been assessed in clinical research studies. They may also present in later life with neurodegeneration, such as those exhibiting parkinsonism in 2 case studies [20, 21].

Forced locomotor tests such as DigiGait are used to highlight gait abnormalities not detectable in a voluntary locomotor test. DigiGait analysis revealed that $Npc1^{+/-}$ mice have altered strides compared with $Npc1^{+/+}$ mice, demonstrating an increase in stride length and duration concomitant with decreased stride frequency. This is a surprising result, as animals with cerebellar ataxia usually present with decreased stride length and duration accompanied by an increase in stride frequency [31, 34, 35]. Given that $Npc1^{+/-}$ mice presented with an intermediate voluntary movement phenotype, we expected these mice to also have moderate gait abnormalities. A possible explanation may be that $Npc1^{+/-}$ mice failed to adjust their gait to the forced locomotion imposed by the treadmill, whereas $Npc1^{+/+}$ mice adapted their walking cadence to the treadmill speed by shortening their stride length with increased stride frequency. This is comparable to the slower and continuous movement of the $Npc1^{+/-}$ mice observed in the open-field test. An alternative explanation based on affective state is also discussed below. Additionally, the forelimbs of $Npc1^{+/-}$ mice have a decreased ataxia coefficient. This result suggested that forelimb control by $Npc1^{+/-}$ mice becomes ataxic with age after a period of compensation for the underlying gait abnormality. It would be of interest to investigate locomotor deficiency in *NPC1* carriers using a sensitive locomotor test such as the GAITRite electronic walkway system [36].

Psychiatric illness is a common clinical presentation often seen in patients with NP-C1 who are > 6 years of age [1, 2, 37, 38]. While our phenotypic assessment focused on motor performance, an anxiety-like phenotype was apparent in $Npc1^{+/-}$ mice. As $Npc1^{+/-}$ mice have comparable ambulatory counts in the total arena of an open-field test to that of $Npc1^{+/+}$ mice, their decreased center tendency is consistent with increased anxiety. Anxiety-like behavior in $Npc1^{+/-}$ mice is also suggested by their longer stride length measured on the DigiGait, a system where an anxious mouse increases its stride length under exposed and anxiogenic conditions, such as that presented by the transparent DigiGait enclosure [32]. The anxiety-like phenotype of $Npc1^{+/-}$ mice thus warrants assessment in additional paradigms, such as elevated plus maze, light–dark exploration, and fear conditioning, to further characterize the nature of their anxiety-like behavior. Nevertheless, our findings suggest that *NPC1* carriers may be more prone to anxiety, which would be consistent with the report of an apparent enrichment of *NPC1* carriers in adult patients with neurological and psychiatric symptoms [23].

The sexual dimorphism we observed in $Npc1^{+/-}$ mice recapitulates previous findings in $Npc1^{-/-}$ mice and patients with NP-C1 [27, 37]. Overall, motor dysfunction and anxiety-like behavior were less severe in female $Npc1^{+/-}$ mice. Cholesterol is the precursor of steroid hormones, including sex hormones. Therefore, sexual dimorphism in $Npc1^{+/-}$ mice may be the result of partial loss of cholesterol regulation and metabolism affecting sex hormone production. In particular, decreased testosterone production has been proposed to underlie the greater loss in motor function in common between male $Npc1^{+/-}$ and $Npc1^{-/-}$ mice, as well as patients with NP-C1 [27, 37, 39].

We conclude that *Npc1* haploinsufficiency causes neurodevelopmental delay with motor and anxiety phenotypes manifest in early-maturity in mice. This raises the suspicion that *NPC1* carriers, who are generally regarded as clinically unaffected, may be predisposed to subtle locomotor impairments that may become apparent as neurodevelopmental delay, and as yet have not been assessed in clinical research studies. They may also present in later life with neurodegeneration, such as those exhibiting parkinsonism in 2 case studies [20, 21]. It is noteworthy that haploinsufficiency of *GBA1*, the gene implicated in another lysosomal storage disorder, Gaucher disease, is a major genetic risk factor for Parkinson's disease [40–42]. Similarly, *NPC1* haploinsufficiency may be a genetic risk factor for Alzheimer's disease, where *NPC1* polymorphisms are associated with risk [43], and *Npc1* haploinsufficiency accelerated formation of neuropathology in an Alzheimer's disease mouse model [44].

Conclusions

Our study has provided proof of principle that *NPC1* haploinsufficiency may cause an attenuated heterozygous disease phenotype. Therefore, *NPC1* haploinsufficiency has the potential to add to the disease burden of about 0.66 % of the population who are pathogenic *NPC1* allele carriers. Further studies will determine whether carriers could benefit from increased medical vigilance and secondary prevention with a drug treatment such as miglustat. $Npc1^{+/-}$ mice may be useful for testing such potential therapeutics.

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