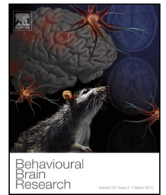
Behavioural Brain Research 292 (2015) 432–442

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr



Research report

Heterozygous L1-deficient mice express an autism-like phenotype

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HIGHLIGHTS

- We investigate the behavior of mice with one copy of the L1 gene inactivated.
- Subjects are socially impaired, exhibit repetitive behaviors, and aversion to light.
- Subjects express normal levels of anxiety, motor abilities, and spatial learning.
- Reduced expression of L1 might contribute to the development of autism.

ARTICLE INFO

Article history:
Received 25 February 2015
Received in revised form 19 May 2015
Accepted 22 May 2015
Available online 14 June 2015

Keywords:
L1CAM
Social behavior
Mice
Autism
Spatial learning

ABSTRACT

The L1CAM (L1) gene encodes a cell adhesion molecule that contributes to several important processes in the developing and adult nervous system, including neuronal migration, survival, and plasticity. In humans and mice, mutations in the X chromosome-linked gene L1 cause severe neurological defects in males. L1 heterozygous female mice with one functional copy of the L1 gene show complex morphological features that are different from L1 fully-deficient and wild-type littermate mice. However, almost no information is available on the behavior of L1 heterozygous mice and humans. Here, we investigated the behavior of heterozygous female mice in which the L1 gene is constitutively inactivated. These mice were compared to wild-type littermate females. Animals were assessed in five categories of behavioral tests: five tests for anxiety/stress/exploration, four tests for motor abilities, two tests for spatial learning, three tests for social behavior, and three tests for repetitive behavior. We found that L1 heterozygous mice express an autism-like phenotype, comprised of reduced social behaviors and excessive self-grooming (a repetitive behavior also typical in animal models of autism). L1 heterozygous mice also exhibited an increase in sensitivity to light, assessed by a reluctance to enter the lighted areas of novel environments. However, levels of anxiety, stress, motor abilities, and spatial learning in L1 heterozygous mice were similar to those of wild-type mice. These observations raise the possibility that using molecules known to trigger L1 functions may become valuable in the treatment of autism in humans.

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1. Introduction

The glycoprotein L1 is a cell adhesion molecule that contributes to neuronal cell migration and survival, neuritogenesis, axon guidance, myelination, and synaptic activity and plasticityduring development and in adults [1–6]. The importance of L1 is evidenced by the severe neurological defects consequent to mutations in its gene. Mutations in the L1 gene in humans can produce the “L1 syndrome”, a severe and rare neurological disorder characterized by mental retardation, dilated cerebral ventricles, hypoplasia of the corticospinal tract, and agenesis of the corpus callosum [7], as well as Hirschsprung's disease [8]. Similarly, mice carrying null mutations in the L1 gene are hydrocephalic (to varying degrees depending on the genetic background) and have abnormal development in the projection of the corticospinal tract and in axons of the corpus callosum [9–11]. In addition, mice with developmentally delayed ablation of the L1 gene under the control of a neuron-specific promoter are impaired in learning and memory [12].

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http://dx.doi.org/10.1016/j.bbr.2015.05.040
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The gene coding for L1 is located on the X chromosome. Owing to the random inactivation of genes on the X chromosome, female carriers of L1 mutations are heterozygous at the level of the L1-expressing cells, in that each cell expresses either the normal or the mutated gene. In humans, heterozygous carriers (L1+/−) of the L1 syndrome usually show a mild phenotype, being thus very different from the severe symptoms of L1-deficient (L1-/y) males [13]. And in mice, contrary to what one would expect, heterozygous females show a complex set of morphological features that are not intermediate between wild-type (L1+/+) and fully L1-deficient (L1-/y) mice [14].

Two features in L1 heterozygous female mice observed by Schmid et al. are of special relevance: (1) L1 heterozygous mice have a higher density of neurons, but not astrocytes, in the motor-sensory cortex relative to their wild-type littermates [14]. This suggests that L1 might modulate motor functions, a possibility that is supported by the observation that application of L1 to cultures of motor neurons prevented their death [15]. (2) Compared to wild-type mice, heterozygous females express a larger volume of the corpus callosum, a tendency to larger sizes of cortices and hippocampi, and, at the microscopic level, a higher density of neurons in the cortex and basal ganglia of both young and adult mice [14]. Furthermore, apoptosis was reduced in L1 heterozygous mice at early postnatal age, implying that not solely the density, but the overall number of neurons is increased in L1 heterozygous mice. Interestingly, increased neuronal density and growth during development has been described in autistic persons [16,17]. And in a case study, a boy with a mutation in the L1 gene presented autistic features with several stereotyped movements of hands and upper limbs [18].

To our knowledge, there are only a few studies measuring behavioral phenotypes in L1-deficient mice. Regarding L1 heterozygous mice, no systematic behavioral investigation has ever been carried out. Here, we designed a study to begin to fill this gap. Given the complexity and novelty in their neuroanatomical traits which are reminiscent of traits of autistic persons, we believe that an exploration of the behavior of constitutively L1-deficient heterozygous female mice is critical to gain more insights into the phenotypes of human female carriers with L1 null mutations.

Here, we investigated the behavior of L1-deficient heterozygous mice by performing a battery of tests in five different categories: anxiety and stress, motor abilities, spatial learning (and spatial learning under stress), social behavior, and repetitive behaviors. We chose/created these tests aiming for functional specificity, so they could be diagnostic of quantitative abnormalities and be easily replicated by other researchers. In particular, we hypothesized that the L1 heterozygote females would exhibit motor impairments as well as autism-like phenotypes of impaired social behavioral and increased repetitive behaviors.

2. Materials and methods

2.1. Animals

The constitutively L1-deficient heterozygous mouse line used in our study was created from animals with an insertion of a tetracycline-controlled transactivator into the second exon of the L1 gene, and then backcrossed for at least 10 generations onto a 129/SvJ background [9,10]. We tested nine female L1 heterozygous (L1−/+) mice and seven female wild-type (L1 +/+) littermate controls, weighing 23–37 g (weight did not differ significantly between the groups). At the start of the study, animals were four months old and were housed individually in a Plexiglas shoebox style cages in a controlled room under SPF conditions and a 12:12 h dark/light cycle with food and water ad libitum. During this time, we handled the mice daily (90 sec/day) for two weeks.

2.2. Behavioral tests

We assessed the animals' behavior with tests in five different categories: 5 tests of anxiety and stress, 4 tests in motor abilities, 2 tests in spatial learning, 3 tests in social behavior, and 3 tests in repetitive behaviors. This diversity of tests was designed to allow a broad spectrum screening. We applied the tests in the same order as reported below, with an interval of about 2 days between each test.

2.2.1. Anxiety, stress, and light responsivity

2.2.1.1. Open field. The open field test is a commonly used test of anxiety, where mice are allowed to explore a novel (typically stress-inducing) open space (for a review on the topic, see Prut and Belzung) [19]. Here, we used a 46 × 46 cm box with 20 cm high walls of white Plexiglas as the open field. The floor of the box was divided with tape into a 6 by 6 grid pattern (7.65 cm for each square), resulting in 20 squares next to the outer walls of the field (i.e., "walled squares"), and 16 squares in the interior of the field (i.e., "center squares"). The box was located in a brightly lit room (250 lx) in order to make the center squares even more anxiety-producing, as mice are averse to open spaces and bright lights. We placed the animals in the center of the box and allowed them to explore it for 5 min, while video recording for later scoring. As the measure of anxiety/stress reactivity, we used the relative time (in percentage) spent in the center squares. Lower numbers indicate more anxiety/stress reactivity.

2.2.1.2. Elevated plus-maze. The elevated plus-maze is a commonly used test of anxiety or stress reactivity where, similarly to the open field test, mice are allowed to explore stress-inducing open spaces (for a review, see Hogg) [20]. Here, we used a maze made of grey Plexiglas in the form of a "plus" shaped platform, 30 cm above the ground, and with four arms (each 4.4 cm wide, 28 cm long). Two opposing arms of the maze were enclosed by 8 cm high, grey Plexiglas walls, and the other two arms were open. The maze was located in a brightly-lit room (250 lx) in order to increase the difference in aversiveness between open and closed arms, as mice are averse to open spaces and bright lights. We placed the mice in the center of the maze facing an open arm and allowed them to explore for 4 min, while video recording for later scoring. As the measure of anxiety/stress reactivity, we used the relative time (in percentage) spent on the open arms. Lower numbers indicate more anxiety/stress reactivity.

2.2.1.3. Light/dark box. The light/dark box test is commonly used to measure anxiety, where mice are allowed to choose between staying in a dark compartment or in a brightly lit, aversive compartment (for a review, see Bourin and Hascoët) [21]. Here, we used a 56 × 15 × 10 (length × width × height) Plexiglas chamber divided into two equal sized compartments (28 cm in length). One compartment was the "dark side", black and lit at 10 lx, and the other compartment was the "light side", white and lit at 300 lx. The whole chamber had a covering lid and a center wall with a 3 cm opening connecting the dark and light sides. We started animals in the black side and observed their behavior for four minutes. As the measure of anxiety/stress reactivity, we used the time spent on the light side. Lower numbers indicate more anxiety/stress reactivity.

2.2.1.4. Light gradient. In order to assess if the results from the light/dark Box test were a reflection of differences in light sensitivity (as opposed to differences in anxiety and stress), we tested the mice in the light gradient test. The apparatus was a thin gray

Plexiglas plank, 204 cm in length without walls, and divided into six sections (36 cm in length) with differing light intensities. Three sections were considered the dark sections and the other three were considered the light sections. The dark sections were lit at 4 lx (section 1), 6 lx (section 2), and 10 lx (section 3). The light sections were lit at 34 lx (section 4), 300 lx (section 5), and 3860 lx (section 6). In both cases, there was a gradual transition of the light intensities between the sections. A small, black square box was placed in the center of the apparatus (at the intersection of sections 3 and 4). We first placed mice inside this box, and then removed it, and allowed animals to explore the entire light/dark plank for 4 min. We measured the total amount of time the animals spent in the dark sections (sections 1–3) and the light sections (sections 4–6), and used the time spent on the light section as the measure of anxiety/stress reactivity (and/or light sensitivity). Lower numbers indicate more anxiety/stress reactivity or light aversion.

2.2.1.5. Post-shock reactivity. We used the post shock reactivity test to measure the stress reaction of animals after a shock presentation. The apparatus was a 16.5 × 26.5 × 20 cm box with a 5 mm steel grid. After 6 min in this chamber, we measured the amount the animals moved (in cm) during the 5 s after the initiation of a 0.6 mA, 500 ms scrambled foot shock. Higher numbers indicate more shock reactivity.

2.2.2. Motor abilities

2.2.2.1. Balance beam. The balance beam test measures motor coordination. We placed mice on a 40 × 7 × 2 cm (length × width × height) balance beam suspended 30 cm above the ground. The beam was explicitly designed so that animals do not typically fall from it. As movement is presumed to interact with balance, we measured the distance traveled across the beam for 5 min.

2.2.2.2. Balance pole. The balance pole test, like the balance beam test, measures motor coordination. We placed the animals on a platform atop a 30 cm high, 4 mm diameter, vertical rod coated with black rubber. We measured the latency to fall off the pole (an index of balance). We placed the mouse back to its home cage after falling or after 2 min; whichever came first.

2.2.2.3. Screen hang. The screen hang test measures paw strength. We placed animals on the underside of a wire mesh screen (7 mm grids) slanted at 40° from vertical and suspended 24 cm from the ground. We measured the distance moved in grids before falling from the screen. We placed the mouse back to its home cage after falling or after 3 min; whichever came first.

2.2.2.4. Rod hang. The rod hang test, like the screen hang test, measures paw strength. We hung the animals by their front paws from a 4 mm black rubber coated rod suspended 30 cm above the ground. We measured the latency until the mice dropped from the rod.

2.2.3. Spatial learning

2.2.3.1. Spatial water maze. The spatial water maze (or morris water maze) test requires mice to locate a submerged platform in a pool of opaque water from which they are motivated to escape. Without distinct intramaze cues, animals' performance in this maze is highly dependent on spatial cues located outside the pool [22]. Animals tend to improve their ability to find the platform (decreasing their path length), despite entering the pool from different locations at each trial.

The apparatus consisted of a round pool (140 cm in diameter and 56 cm deep) filled with water opaqued by nontoxic black paint. A hidden black platform (14 cm diameter) was located 1.5 cm under the water. The pool was inside a dark room, and surrounded by three bright, but differently shaped lights, as well as by drawings on the walls, all of which served as visual cues.

Prior to training, mice were acclimated to the maze by being confined to the pool's platform (that was surrounded with a clear Plexiglas cylinder) for 4 min. During initial training, we started the animals from one of three positions for each trial such that no two subsequent trials started from the same position. The platform was always in the same position. A mouse was said to have successfully located the platform when it remained on the platform for 5 s. After locating the platform or swimming for 90 s, we kept animals on the platform for 5 s, after which we removed them for a 10 min intertrial interval inside a holding box. Each animal completed three days of training with 6 trials per day, and was recorded for later scoring. We used the path length covered by each animal from trials 2 to 6 as the measure of spatial learning.

2.2.3.2. Radial arm maze under chronic stress. Similar to the spatial water maze test, the radial arm maze test requires that a mouse to use spatial cues (distributed around the maze) to guide its search. In order to efficiently find food, animals have to maintain a memory of arms that have been visited within a trial.

The maze was made of grey Plexiglas with a central area (15 cm in diameter) where eight arms evenly radiated out (40 cm long and 4.5 cm wide), with no walls. The end of each arm had a depression containing a piece of food (14 mg of Dustless Precision pellets, Bio-Serv). The maze was located in a room with a variety of visual cues, including architectural details (like walls, shelves, and ceiling), light strings, and geometric shapes affixed to the walls.

We started the test by placing an animal in the central area with all arms baited. The mouse was allowed to move freely until it retrieved the last of the eight pieces of food or until 15 min have passed; whichever came first. We measured the streak of correct choices an animal made. A choice was counted as hind paws passing 1/4 of an arm's length (same criterion we used in past research [23,24]). We administered one trial per day, for a total of four days. We used the number of consecutive correct choices for each animal from trials 2 to 4 as the measure of spatial learning.

According to Merino, Cordero, & Sandi (2000), intense stress might play a role in the observed regulation of L1 expression during spatial learning. Because of this potential interaction between L1, stress, and spatial learning, we chronically stressed all animals prior to and during the radial arm maze test. We placed animals for 1 h in a 50 mL Eppendorf tube daily during one week before the Radial Arm Maze test started, and then during each day of test. This allowed us to test for differences in spatial learning under (relatively) normal conditions (spatial water maze) and under chronic stress (radial arm maze).

2.2.4. Social behavior

Impairments in social behavior are a hallmark of the autism-spectrum [26]. Here, we used 3 tests to assess mice's social behavior in hope that they could shed a light on potential effects of L1 on autism.

2.2.4.1. Sociability. The social test chamber is a common way to analyze the sociability of mice, and has been used many times for the detection of autistic-like traits [26–28]. Here, we used a clear Plexiglas box that measured 61 cm (length) by 40 cm (width) by 21 cm (height) as the social chamber. This box was open at the top and the bottom surface was black. The box was divided into three chambers, where the center chamber was connected to the right and left chambers by remotely operated vertical doors.

First, we acclimated the animals to the apparatus, to help them adjust to the environment of this test. On the acclimation day, the animals spent 10 min in the box with no other animals present, and the animals were allowed free access to all chambers.

On the first day of testing, a new, C57BL/6 male mouse was placed on either the left or right side of the box, and was enclosed in a small, silver-colored cylindrical cage (constructed of stainless steel bars) that measured 9.5 cm (diameter) and 15.5 cm (height). To make sure that the animals did not harm the C57BL/6 male, a stainless mesh (0.6 cm grid) covered the cylindrical cage. An experimental animal was subsequently placed in the middle section of the box for 2 min with the doors to the left and right sides closed. After this, the doors were removed and the animal was allowed to travel freely in the box for 10 min. One side was designated as the "C57BL/6 male" side (it contained the target animal in the cylindrical cage) and the other side was designated as the "empty" side (with no animal present). We alternated the location of each side between experimental animals to minimize the smell of the target animal (or other cues) coming from only one particular direction, as well as to minimize, across groups, any spatial preferences. For the first animal tested, the C57BL/6 male was on the left side, then, for the second animal, the C57BL/6 male was on the right side, then, for the third animal, the C57BL/6 male was on the left side, and so on. For the measure of sociability, we used the time an animal spent on each side of the box.

2.2.4.2. Resident–intruder. As a second measure of social behavior, we used the resident–intruder test (for examples, see Jamain et al.; Winslow and Miczek) [29,30]. We tested animals twice: first with intruders being males of the C57BL/6 strain, and then with intruders being wild-type females from the L1 mutant strain of the same age as the test animals. For each test, we placed an intruder inside the cylindrical cage described above, and placed the cylinder in the subject's home cage. Four 5 min trials (of each type), were administered during the middle portion of a single light cycle, and the trials were separated by 120 min. The two tests (female or male) were separated by 7 days. For the measure of social behavior, we used the duration of contact by the resident, defined as its snout getting within 1 cm of the cylindrical cage.

2.2.4.3. Nest building. Nest construction is an important indicator of social and reproductive behavior [31,32]. For this reason, it is a frequently used test in research on autism-like traits in mice [26,28].

In this test, mice were housed individually in new home cages. On the first day of the test, we placed a 2 cm by 2 cm cotton (nest material) square on the outside of each animal's cage underneath the water bottle. The water bottle and food were in their regular positions outside the cage. This arrangement was maintained overnight (for 12 h). The following day, we weighed the cotton not used by the animals, and used it as a measure of the propensity for nest building.

2.2.5. Repetitive behaviors

Repetitive, stereotyped behaviors are characteristic of the autism-spectrum [26]. Consequently, we assessed three distinct repetitive behaviors.

2.2.5.1. Induced self-grooming. Self-grooming is a behavior that mice engage in to maintain their appearance and comfort, and its excessive occurrence has been observed in animal models of obsessive-compulsive disorders [33] and autism [34]. Here, we amplified the natural tendency to groom by spraying water at the subjects—a technique used before by others [26]. We defined self-grooming behavior as all the elliptical, unilateral, and bilateral strokes each animal made. Elliptical strokes are defined as asymmetric movements of a mouse's forepaws over the nose and muzzle; unilateral strokes are defined as alternating strokes of the forepaw across the mouse vibrissae and eye; and bilateral strokes are defined as forepaw strokes that begin behind the ears and pass over the entire face. We performed this test in each animal's home cage for five minutes after delivering one gentle mist of water to the animal's back side from a distance of approximately 10 cm. For the measure of repetitive behavior, we used the total time (in second) animals spent self-grooming.

2.2.5.2. Marble burying. Mice will obsessively bury marbles (and other similar objects) that are introduced into their home environment, and this tendency can be used to assess repetitive and compulsive behaviors [35].

For this test, we used standard, shoebox-style rat cage rather than the typical mouse cage, and filled it approximately 5 cm deep with new bedding. Each cage had 24 marbles, symmetrically placed in rows of 4 and columns of 6. We placed the animals individually in the cage and left them for 30 min. After this time, we counted the number of marbles buried underneath the rat cage's bedding. A marble was considered buried if more than 2/3 of it was buried beneath the bedding surface.

2.2.5.3. Digging. Digging is a natural behavior that, similar to the artificial behavior of marble burying, can be used to assess repetitive and compulsive behaviors [35].

In this test, we placed the animals in a typical, clear mouse shoebox cage filled approximately 5 cm deep with bedding. We recorded the total duration of a digging behavior for 5 min.

2.3. Statistical analyses

All behavioral scoring and assessments were made by an experimenter who was blind to the animal's group assignment. In order to evaluate the results, we performed two-tailed t-tests, between subjects ANOVAs, and repeated measures ANOVAs using SPSS 21. The data in figures and text are expressed as means ± SEM. We considered a p value at or below 0.05 to indicate a significant difference.

3. Results

In all cases, we describe the comparison between L1 heterozygous (L1+/−) and wild-type (L1+/+) female animals. Tests were categorized as: anxiety and stress, motor abilities, spatial learning, social behavior, and repetitive behaviors.

3.1. Anxiety and stress

Results for the Open field test can be seen in Fig. 1A. We used the relative time spent in the center of the open field as measure of anxiety and/or stress reactivity, and found that heterozygous animals did not differ significantly from wild-type animals, $t(14) = 0.797$, $p = 0.439$. We found a similar pattern for the elevated plus-maze test (Fig. 1B), with heterozygous animals not differing significantly from wild-type animals in the relative time spent in the open arms, $t(14) = 0.106$, $p = 0.917$.

For the light/dark box test (Fig. 2A), we used time spent in the light side as a measure of anxiety and/or stress reactivity to the light. Heterozygous animals spent significantly less time in the light side than wild-type animals, $t(14) = 2.655$, $p = 0.019$. A somewhat different pattern occurred in the light gradient test. For the time spent in the light side in that test (Fig. 2B), we found that heterozygous animals did not differ significantly from wild-type animals, $t(12) = 0.175$, $p = 0.864$. However, for the latency to enter each gradient (dark or light), there was a significant interaction between group (wild-type or L1 heterozygous mice) and gradient, $F(1,12) = 4.62$, $p = 0.05$ (from a mixed design ANOVA, Fig. 2C), where it was observed that L1 heterozygous mice were quick to enter the dark gradient but slow to enter the light, whereas wild-type animals exhibited the opposite pattern of behavior.

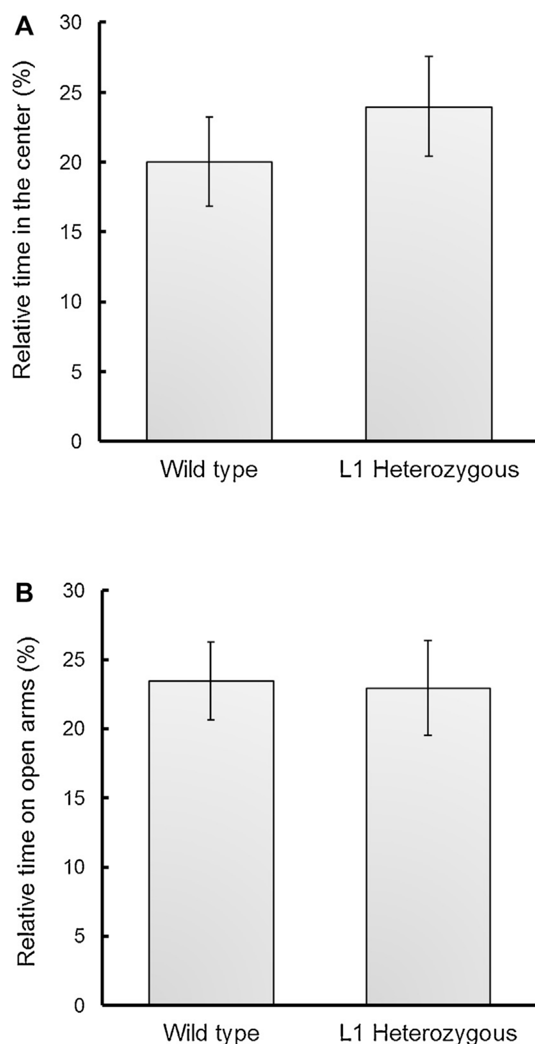


Fig. 1. (A) Average relative time spent in the center (%) of the open field for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. (B) Average relative time spent on the open arms (%) of elevated plus-maze for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

For analysis of post-shock reactivity, we used the amount of movement during the 5 s after the shock as a measure of stress reactivity (Fig. 3). Heterozygous animals did not differ significantly from wild-type animals, $t(12) = 1.519$, $p = 0.155$, although there was a tendency for the heterozygous mice to move less.

3.2. Motor abilities

Results for the two tests measuring motor coordination, the balance beam and balance pole tests, are shown in Fig. 4A and B. In the balance beam test, the number of beam crossings did not differ significantly between heterozygous and wild-type animals, $t(14) = 0.664$, $p = 0.518$. No animals from either group fell from the beam. Similarly, in the balance pole test, the latency for animals to fall did not differ significantly between heterozygous and wild-type animals, $t(14) = 1.536$, $p = 0.147$.

Results for the two tests measuring paw strength, the screen hang and rod hang tests, are shown in Fig. 5A and B. In the screen hang test, the number of grid crossings did not differ significantly between heterozygous and wild-type animals, $t(14) = 1.919$, $p = 0.076$. Similarly for the rod hang test, the latency for animals to

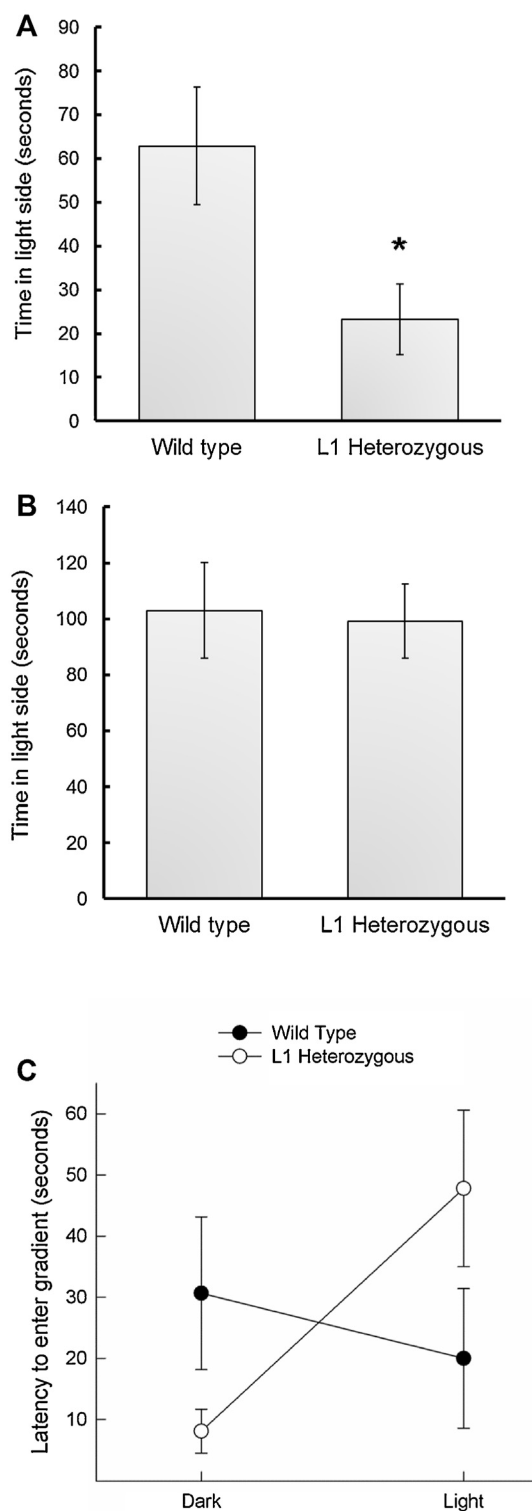


Fig. 2. (A) Average time spent in the light side of the light/dark box for wild-type and L1 heterozygous mice. L1 heterozygous animals spent significantly less time in the light side than wild-type animals. (B). Average time spent in the light side of the light gradient for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. (C). Average latency to enter each gradient (dark or light) for wild-type and L1 heterozygous mice. There was a significant interaction between group and gradient. Brackets indicate standard error of the mean.

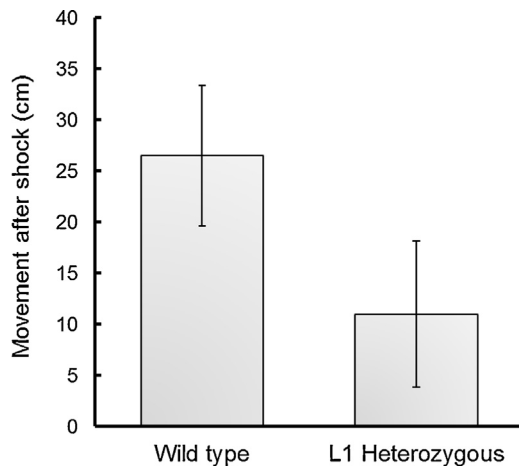


Fig. 3. Average movement (in cm) during 5 s after shock in the post-shock reactivity test for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

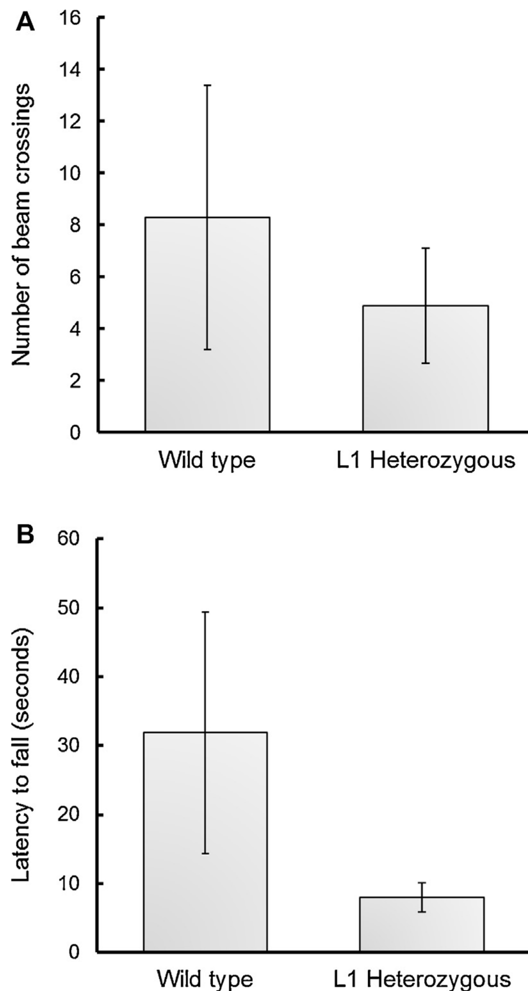


Fig. 4. (A). Average number of beam crossings (one side of the beam to the other) in the balance beam for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. (B). Average latency to fall (in second) from the balance pole for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

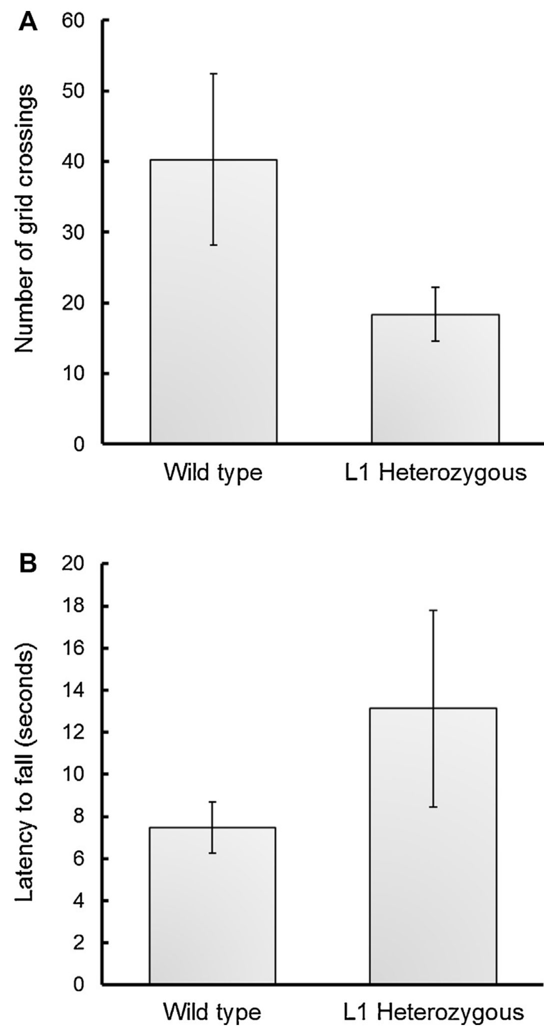


Fig. 5. (A). Average number of grid crossings in the screen hang for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. (B). Average latency to fall (in second) from the rod hang for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

fall did not differ significantly between heterozygous and wild-type animals, $t(14) = 1.043$, $p = 0.315$.

3.3. Spatial learning

Results for the spatial water maze test are shown in Fig. 6A. We used the distanced traveled (path length) by each animal from trials 2 to 6 as a measure of spatial learning. A repeated measures ANOVA showed that heterozygous animals did not differ significantly from wild-type animals across the trials, $F(1,14) = 0.042$, $p = 0.841$. The mice showed a similar pattern for spatial learning under chronic stress in the radial arm maze test (Fig. 6B). A repeated measures ANOVA showed the number of consecutive correct choices from trials 2 to 4 did not differ significantly between heterozygous and wild-type animals, $F(1,14) = 0.018$, $p = 0.896$.

3.4. Social behavior

Regarding the sociability test (Fig. 7A), L1 heterozygous animals explored the side with the C57BL/6 male significantly less than the empty side, $t(16) = 3.29$, $p = 0.005$. In comparison with wild-type, L1 heterozygous animals spent significantly less time on the C57BL/6 male side, $t(14) = 2.93$, $p = 0.011$. For the time spent on

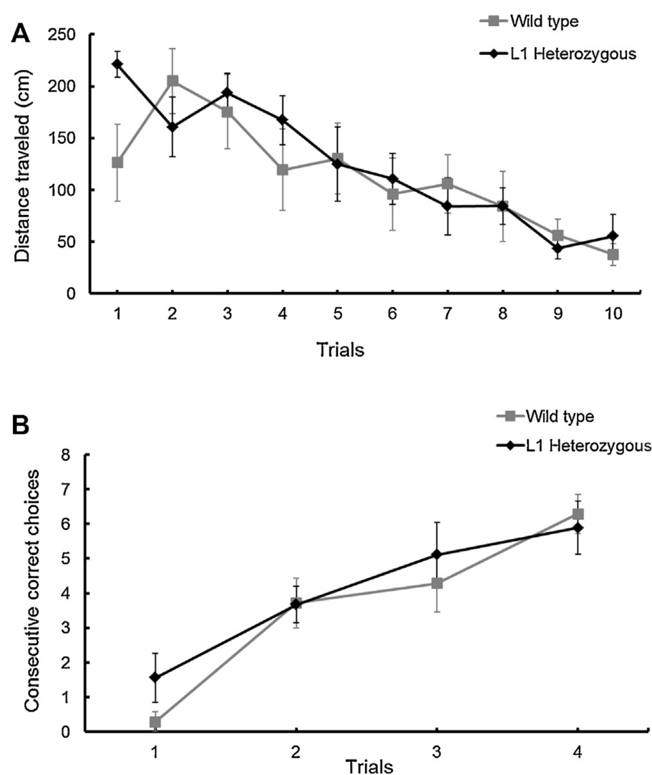


Fig. 6. (A). Average distance traveled (path length, in cm) in the Water Maze for wild-type and L1 heterozygous mice across 10 trials. There were no significant differences between the two groups. (B). Average streak of correct choices in the radial arm maze (under chronic stress) for wild-type and L1 heterozygous mice across 4 trials. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

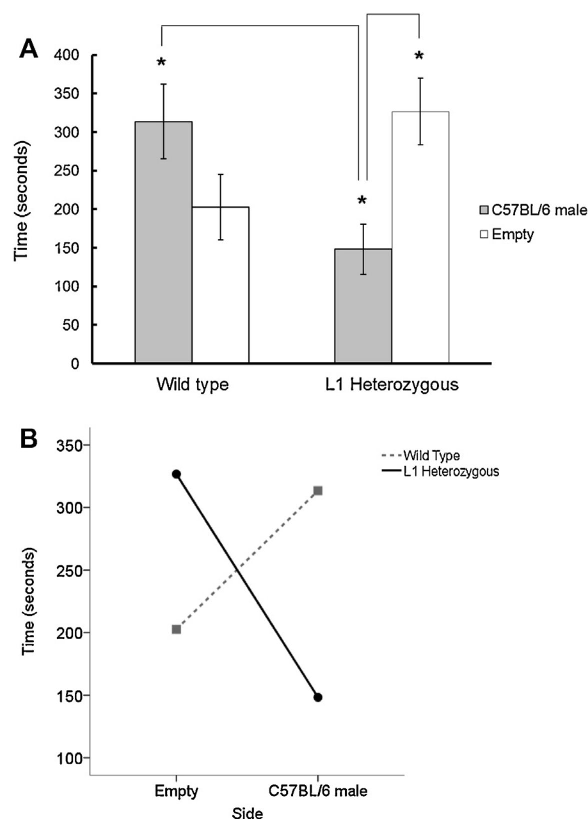


Fig. 7. (A). Average time spent (in second) in the sociability test on the empty side and on the side with a C57BL/6 male mouse. L1 heterozygous animals explored the target side significantly less than the empty side. In the comparison with wild-type, heterozygous animals spent significantly less time on the side with a mouse. Brackets indicate standard error of the mean. (B). Significant crossed interaction between the side of the box (empty or male mouse) and group (wild-type or L1 heterozygous).

the empty side, there were no significant differences, $t(14) = 2.00$, $p = 0.065$, although heterozygous mice's lower values approached significance. An ANOVA showed a significant interaction between the side (target or empty) and the group (heterozygous or wild-type), $F(1,28) = 11.92$, $p = 0.002$. Furthermore, this interaction was crossed, as seen in Fig. 7B, with L1 heterozygous animals having the highest values of exploration for empty and lowest for C57BL/6 male, while wild-type animals had the lowest values for empty and highest values for C57BL/6 male.

In the resident–intruder test, when the intruder was a male, a paired-samples t tests showed reduced exploration from the first to the last (fourth) trials for both heterozygous animals, $t(8) = 2.26$, $p = 0.053$ (borderline significance), and wild-type animals, $t(6) = 3.79$, $p = 0.009$, meaning that both strains adapted to the presence of the intruder (Fig. 8A). A repeated measures ANOVA further supported this, with a significant effect of trial on the duration of exploration in both strains, $F(3,42) = 5.19$, $p = 0.004$. Surprisingly, however, there were no significant differences between heterozygous and wild-type animals across the trials, $F(1,14) = 1.33$, $p = 0.270$. When the intruder was a female, a paired-samples t tests showed no change in exploration from the first to the last (fourth) trials for either heterozygous animals, $t(8) = 1.50$, $p = 0.173$, or wild-type animals, $t(6) = 1.01$, $p = 0.350$, meaning that neither strain habituated to the intruder (Fig. 8B). A repeated measures ANOVA further showed that there was no significant effect of trial on the duration of exploration in both strains, $F(3,42) = 1.23$, $p = 0.311$. And once again, there were no significant differences between heterozygous and wild-type animals across the trials, $F(1,14) = 1.18$, $p = 0.295$. Between the two tests, wild-type animals interacted significantly more with female intruders than male intruders across the 4 trials, $F(1,12) = 15.29$, $p = 0.002$. For L1 heterozygous animals, however, there were no significant differences, $F(1,16) = 2.18$, $p = 0.159$, suggesting that L1 deficient animals were less sensitive to the sex of the intruder.

In the nest building test, we used the percentage of cotton used by the mice when building the nest as a measure of social behavior (Fig. 9). Heterozygous animals did not differ significantly from wild-type animals, $t(14) = 0.176$, $p = 0.863$.

3.5. Repetitive behaviors

For the induced self-grooming test, we used the duration of self-grooming after a spray of water as a measure of repetitive behavior (Fig. 10A). L1 heterozygous animals groomed for significantly longer periods of time than wild-type animals, $t(14) = 2.656$, $p = 0.019$. However, the same pattern did not occur for the other two tests of repetitive behaviors (below).

In the marble burying test (Fig. 10B), the number of marbles buried did not differ significantly between heterozygous and wild-type animals, $t(14) = 1.392$, $p = 0.186$. Likewise, in the digging test, there were no differences across the two groups (Fig. 10C), $t(14) = 0.267$, $p = 0.793$.

4. Discussion

We contrasted the behavior of L1-deficient heterozygous female mice to their wild-type female littermates, and hypothesized that the L1 heterozygous mice would exhibit motor impairments as well as an autism-like phenotype. Several observations were consistent with this latter possibility. L1 heterozygous mice interacted signif-

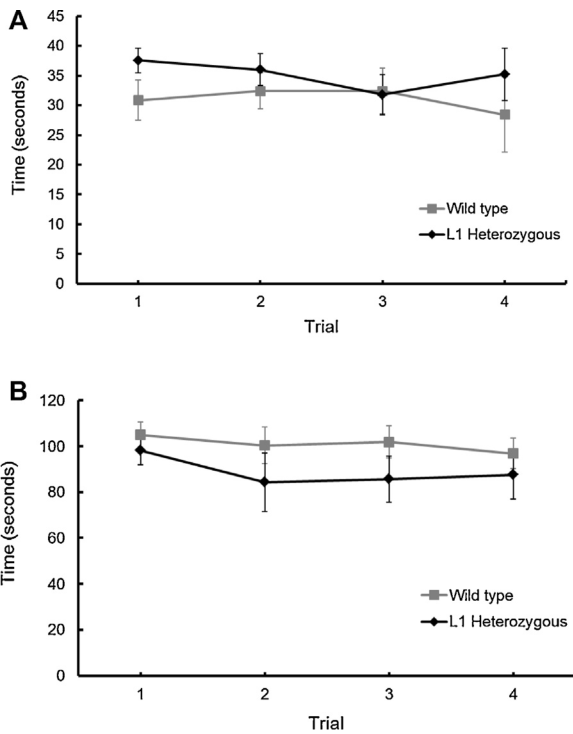
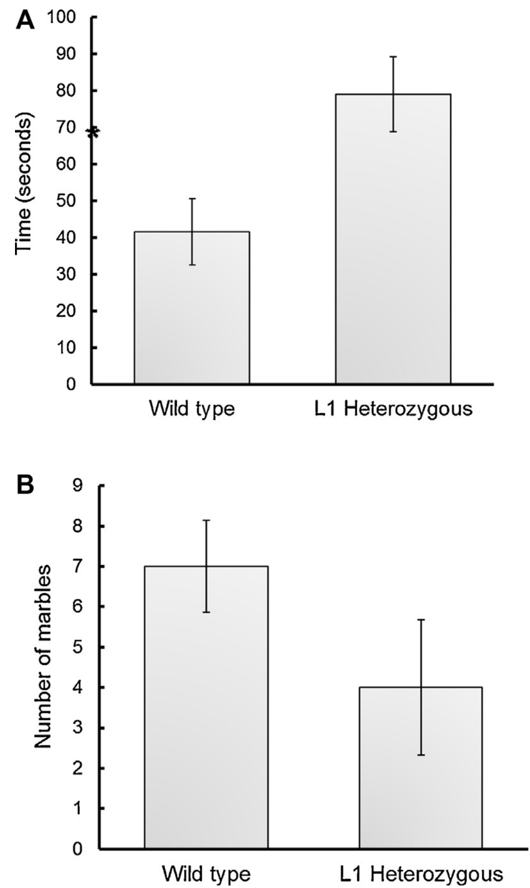


Fig. 8. (A). Average duration of contact (in second) in the resident–intruder test with males as intruders for wild-type and L1 heterozygous mice across 4 trials. There were no significant differences between the two groups. (B). Average duration of contact in the resident–intruder test with females as intruders for wild-type and L1 heterozygous mice across 4 trials. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

Fig. 9. Average relative amount of cotton used (%) during the nest building test for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. Brackets indicate standard error of the mean.



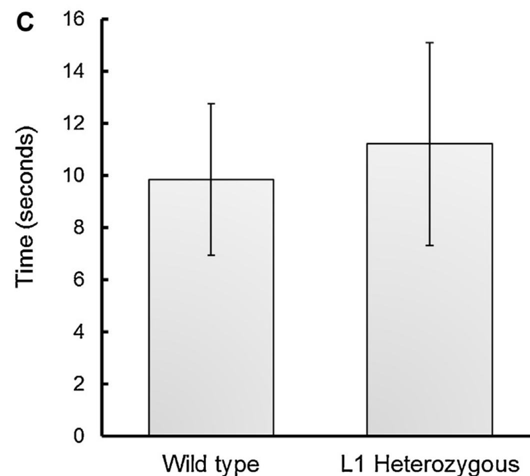


Fig. 10. (A). Average time spent self-grooming (in second) after a spray of water for wild-type and L1 heterozygous mice. L1 heterozygous animals groomed for significantly longer periods of time than wild-type animals. (B). Average number of marbles buried in the Marble Burying test for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. (C). Average time spent digging (in second) for wild-type and L1 heterozygous mice. There were no significant differences between the two groups. Brackets indicate standard error of the mean.

icantly less with another mouse in the sociability test, a standard test of autism-like behaviors in mice. Likewise, L1 heterozygous mice appeared averse to brightly lit novel environments, although in general (absent distinct light disparities), their exploratory tendencies did not differ from wild-type animals. The L1 heterozygous mice also self-groomed relatively more, a repetitive behavior typical in autism. These findings complement at the behavioral level the neuroanatomical results from Schmid et al. where L1 heterozygous females had a higher density of neurons in the cortex, as well a higher number of neurons (due to reduced apoptosis) at early postnatal age. Increased number and density of neurons seems to be a common trait in cases of autism in humans [16,17], and, with our results here, something similar appears true for L1 heterozygous female mice. Regarding motor skills, however, there were no impairments due to the gene deletion. We were surprised to find that L1 heterozygous mice did not differ from wild-type mice, since previous neuroanatomical data from Schmid et al. [14] suggested that motor impairments might arise as a consequence of gene deletion.

For a more comprehensive analysis, we discuss in detail below the results in all five different behavioral categories assessed here: anxiety and stress, motor abilities, spatial learning (and spatial learning under stress), social behavior, and repetitive behaviors.

4.1. No deficits in anxiety, stress, motor abilities, and spatial learning

Regarding anxiety and stress, there were no differences in the performance between L1 heterozygous and wild-type animals in the open field, elevated plus-maze, and post-shock reactivity tests. There was, however, a difference in the light/dark box test, where L1 heterozygous animals spent significantly less time in the light side of the box. However, because the same general measure (time spent in the light side) was not significant in the light gradient test, this effect may be spurious. It is more plausible, though, that the performance of L1 heterozygous animals reflects an increased sensitivity to light. When measuring the latency to first enter a gradient (dark or light) In the light gradient test, there was a significant interaction between groups and gradient, with L1 heterozygous animals entering the dark side of the gradient more quickly, but taking longer to enter the light side. The opposite pattern was observed for wild-type animals. In a molecular characterization of L1 heterozygous mice, Schmid et al. [14] found that those animals have, compared to wild-type, significantly more cerebral protein synthesis (in fractional leucine uptake) in the sensory cortex as well as increased cerebral blood flow in the visual cortex (and only in the visual cortex, from an analysis of 15 major regions of the brain). Those observations imply that L1 heterozygous animals probably have increased neuronal density/activity in their visual cortex. Hence, the increased light sensitivity in L1 heterozygous mice that our current study suggests is a novel and interesting result that deserves future investigation.

Fransen (1998) found that male mice with no L1 expression show increased anxiety (as indexed by fewer entries into the center of an open field) compared to wild-type mice. However, as a whole, our results on anxiety and stress indicate that animals with constitutive L1 heterozygosity show a pattern of anxiety and stress responsivity closer to wild-type animals than to animals with L1 fully deficient (be it during or after development).

Tests of motor abilities revealed no abnormalities in the performance of the L1 heterozygous animals. This was unexpected, since L1 is known to be a survival factor of motor neurons [15]. Surprisingly, in the study by Schmid et al. [14] L1 heterozygous mice showed a higher neuronal density in the motor-sensory cortex relative to wild-type mice. Regardless, the results reported here suggest that this neuroanatomical difference in L1 heterozygous animals does not have any functional effect on motor behavior (at least in regard to the set of tests employed here). In this regard, it is worth noting that in the present study, swimming speed did not differ between L1 heterozygous and wild-type animals (data not shown), again suggestive of normal motor performance in the L1 heterozygous animals.

Regarding spatial learning, L1 heterozygous animals did not exhibit any deficits in the Spatial Water Maze test. Furthermore, our results in the radial arm maze test under conditions of chronic stress showed that the L1 heterozygous animals learned at a rate comparable to the wild-type animals. This seems, at first glance, to contradict the findings of Merino et al. [25], where higher stress intensity during tests of spatial memory lead to an increase in the expression levels of L1 in rats' hippocampi (a brain region critical for spatial memory). However, these authors also found that levels of L1 were reduced during intermediate values of stress, which could be the case in our stress procedure. Regardless, there is also a possibility of a ceiling effect, with both spatial water maze and the radial arm maze not taxing the mice sufficiently to reveal differences between the groups. Nevertheless, in two standard tests of spatial navigation, under nominally unstressed and chronically-stressed conditions, we found no evidence of a learning deficit in L1 heterozygous animals.

Humans with L1 syndrome as well as mice with the full L1 ablation have distinct, and sometimes severe, cognitive deficits [7,12]. Therefore, our results in spatial learning suggest, once again, a behavioral pattern in L1 heterozygous mice after development very distinct from L1 homozygous mice during/after development. This corroborates the findings of Gallistel et al., who reported no differences in learning tasks between female L1-deficient heterozygous mice and wild-type mice. In that study, heterozygous mice showed normal, wild-type rates of instrumental and classical conditioning, and normal capacity to match investment with reward. However, this does not mean that L1 has no effect on cognitive performance in mice. In the same study, Gallistel et al. [36] found that L1 heterozygous females show an increase in the precision to time interval durations. Also, in a previous study with mice deficient in CHL1, another cell adhesion molecule homologous to L1, we found a widespread effect on working memory duration, although CHL1-deficient mice did exhibit normal learning in a test of spatial navigation [37]. Hence, the possibility that L1 in heterozygosity after the completion of development leads to mild cognitive effects cannot yet be entirely disregarded.

4.2. Deficits in social and repetitive behaviors, and their relevance to autism

In the Sociability test, the L1 heterozygous mice explored the empty side of the apparatus (cylindrical cage only) more than the side of the apparatus containing a mouse (inside a cylindrical cage)—a pattern indicative of avoidance of the conspecific. The opposite pattern of behavior was observed in wild-type animals. Typical social mammals, like mice and humans, tend to interact more with animals of the same species rather than with inanimate objects; the opposite pattern is an indication of behaviors typical of autism, like commonly seen in human children [38,39]. Indeed, the test of sociability is the standard test for autistic-like traits, and has been used to confirm many successful mouse models for autism [26–28]. Importantly, our results on anxiety and stress indicate that the results of the sociability test do not reflect a general impairment, but instead, a specific impairment in social contexts.

In the resident–intruder test, both L1 heterozygous and wild-type animals familiarized (over successive exposures) with their male intruders. While we expected L1 heterozygous mice to interact with the intruder less often, this was not the case. This result seems contradictory with what we obtained in the sociability test. However, those two tests are quite distinct. While the sociability test is a pure test of social behavior (measuring a mouse's preference for socializing or interacting with an inanimate object), the resident–intruder test is influenced by social memory, sexual behavior, and aggressive/defensive behavior, a collection of traits that are not stereotypical of autism in humans. This distinction can be suggested from our data by the fact that when females were the intruders, both L1 heterozygous and wild-type residents did not exhibit a reduction in exploration over the four trials. Also, both wild-type residents interacted far more with females than males, and there was a trend (though not significant) toward the same pattern by L1 heterozygous residents. It is conceivable that all our subjects, being females, had sexual or defensive impulses to avoid male, but not female, intruders. Using other mouse models of autism, El-Kordi et al. found that only mutant males showed a difference in behavior from the wild-type control. Furthermore, most studies using the Resident-Intruder test with females as subjects have only other females as intruders.

We did not find differences between L1 heterozygous and wild-type animals in their propensity for nest building. Though unexpected, the fact that our subjects were females (by necessity) might explain these results. Adult mice are much more likely to build nests when in company of other littermates, and females, in particular, show a high degree of nest building only during maternal phase (during pregnancy and a few days after delivery) [40]. In a study with mouse models for autism, El-Kordi et al. found that only male mutants had a higher proportion of untouched nesting material and built significantly less functional nests. Female mutants showed no differences from wild-type animals, a result consistent with what we observed here.

In addition to deficits in social behavior, increased repetitive behaviors are also a hallmark of the autism-spectrum disorders. In our tests, L1 heterozygous and wild-type animals did not differ in their levels of marble burying and digging. However, the heterozygous animals exhibited higher levels of self-grooming in the induced self-grooming test. Excessive grooming has been observed in animal models of obsessive-compulsive disorders [33] and autism [34]. Regarding sex differences, Schmeisser et al. found in another mouse model of autism that self-grooming is higher in female knockout mice compared to wild-type mice, while males did not differ across groups. The question, then, remains: do L1 heterozygous mice have increased repetitive behaviors in general? In contrast with our tests of digging and marble burying, in our test of induced self-grooming animals had their natural tendency amplified (by water spray). If we assume that L1 heterozygous animals have a milder degree of autistic traits, it could be that their repetitive behavior was only above the threshold of detection in the self-grooming test. In this regard, we observed no obvious differences in self-grooming behavior under baseline condition (i.e., absent the spray of water; data not shown).

For autistic traits as a whole, there are also important considerations regarding the fact that our animals were females (males cannot be heterozygous for L1, since the gene is located on the X chromosome). In humans, males and females greatly differ in the severity and distribution of autistic symptoms [42,43]. Different genetic expression levels between sexes probably play an important role in modulating the expression of autism. Of note, sex differences have been reported in several other mouse models of autism; with mutations in certain genes affecting males more than females [41,44]. This suggests the possibility that the autistic-like behaviors observed here for L1 heterozygous females are overall milder and and of a different pattern than would be the case in males.

5. Conclusions

Taken in combination, L1 heterozygous animals display mild social impairments and higher levels of grooming, and thus may display an autistic-like phenotype. The behavioral screening for other traits (anxiety, stress, spatial learning, and motor abilities) allowed us to gain further insight on the role of L1 in the brain when in heterozygosity (and potential effects of different L1 expression levels), as well as its role during development, which represents a critical period since autism is a developmental disorder, with gene defects probably having an effect early during development [45]. Future studies in humans should investigate if L1 carriers have, like mice, mild autistic-like traits. Future studies in mice should test if an increase in levels of expression of L1 in heterozygous animals after development can lead to a reduction in the autistic-like traits that were observed here. If confirmed, this line of research could become valuable for a potential treatment of autism in humans. For example, agents that lead to more L1 related functions (by triggering beneficial signals in the one dose level of L1 in heterozygosity) such as L1 antibodies, peptides, and small organic compounds, could be administered to L1 heterozygous mice with the hope that these agents will improve autistic-related behavioral abnormalities.

Acknowledgements

This work was supported by grants from the National Institute of Aging(AG022698) and the Office of Naval Research (N000141210873) to L.D.M.

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