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LRRTM1-deficient mice show a rare phenotype of avoiding small enclosures—A tentative mouse model for claustrophobia-like behaviour

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Abstract

The LRRTM family proteins have been shown to act as synaptogenic cell adhesion molecules via interaction with presynaptic neurexins and are associated with neuropsychiatric disorders. LRRTM1-knockout mice have subtle morphological deficits in excitatory hippocampal synapses and were suggested to have impaired cognitive function. Here we report that LRRTM1-knockout mice exhibit an extraordinary phenotype of avoiding small enclosures. In the light–dark box, the knockout mice escape to dark through a standard opening as quickly as wild-type littermates but avoid escaping through a small doorway. While all wild-type mice spontaneously enter a small tube, most knockout mice do not. This apparent aversion to enter narrow space may explain other abnormalities such as increased time in open arms in the elevated plus maze and less visits through a tunnel in the IntelliCage. Moreover, LRRTM1-knockout mice show increased social interaction, reduced nest building and MK801-induced locomotion, and slower swim speed but normal water maze learning. Since LRRTM1 is predominantly expressed in thalamus, hippocampus and limbic cortex, specific synaptic defects in those areas presumably cause these behavioural abnormalities.

Keywords

Anxiety; Leucine-rich repeat; Neurexin; Species-typical behaviour; Social interaction; Claustrophobia

1. Introduction

The LRRTM (leucine-rich repeat transmembrane) family proteins [1] were identified as synaptogenic cell adhesion molecules to organize the molecular composition and thus

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Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2012.10.013.

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functional properties of central synapses [2,3]. Consistent with this idea, LRRTM1-deficient (Lrrtm1-KO) mice have subtle morphological defects in glutamatergic synapses in the hippocampus [2]. Recent studies have identified LRRTMs as ligands of neurexins [4–6], presynaptic adhesion proteins that can interact with several postsynaptic partners (including neuroligins and LRRTMs) [7]. Knockdown experiments suggest functional redundancy between LRRTMs and neuroligins during development and functional divergence upon synapse maturation [8,9]. Neurexin and its binding partners have been implicated in the etiologies of developmental neuropsychiatric disorders, including autism spectrum disorders and schizophrenia [10–12]. *LRRTM1*, in particular, has been associated to human handedness and schizophrenia [13,14].

To investigate the physiological role of LRRTM1, we aimed to carry out a comprehensive screening of behavioural phenotype of the Lrrtm1-KO mice [2]. Behavioural analysis of neurexin-1a-deficient mice was available [15] and behavioural analysis of another mouse line lacking LRRTM1 was reported [3] during the preparation of this manuscript. Therefore, we had the possibility to modify and adapt our standard testing protocols [16] according to the already published data in order to find supporting or contrasting evidence. There has been discussion on the variability and external validity in mutant mouse phenotyping studies [17,18]. Of note, we were able to confirm several similarities between the two independently generated and tested Lrrtm1-KO mouse lines. In addition, our extended analysis revealed an extraordinary and novel phenotype in the Lrrtm1-KO mice expressed as avoidance of small enclosures.

2. Materials and methods

All animal experiments were carried out in accordance with the Guidelines laid down with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the County Administrative Board of Southern Finland (license number ESAVI-2010-09011/Ym-23).

2.1. Animals and housing

Lrrtm1-KO heterozygotes [2] were backcrossed into C57BL/6JOlaHsd (Harlan, UK) for 6–8 generations and heterozygous animals were then intercrossed to obtain Lrrtm1-KO mice and wild-type (WT) littermates. Three cohorts of mice (number of KO females: 7 + 8 + 9, WT females: 10 + 6 + 9, KO males: 6 + 7 + 7, WT males: 3 + 6 + 8) were transferred into behavioural lab at the age of 2 months. The mice were housed in standard polycarbonate cages covered with wire lid (Macrolon, Scanbur A/S, Karlsrunde, Denmark) in groups of 2–5 animals and tested in a battery of behavioural tests at 2.2–4.5 months of age. The bedding (aspen chips, Tapvei Oy, Finland) was changed weekly and a wooden tube and aspen shavings (Tapvei) was provided as an enrichment. The food and water were available *ad libitum*. The animals were maintained under a 12-h light–dark cycle (lights on at 6 a.m.) at relative humidity 50–60% and room temperature 21 ± 1 °C.

2.2. Behavioural studies

A battery of tests applied for behavioural phenotyping is described in detail elsewhere [16]. The order of tests and number of animals used is shown in Table 1. There was an interval of at least 1 day between the different tests. All experiments were carried out between 9 a.m. and 4 p.m. by an experimenter blind to the genotype of the animals.

2.2.1. Video tracking—The mice were video-tracked by Noldus EthoVision XT 8.0 system (Noldus Information Technology, Wageningen, The Netherlands) during the

elevated plus-maze, water maze, sociability, object exploration and forced swim tests. The distance travelled by the subjects and the time spent in pre-defined zones was recorded.

2.2.2. Elevated plus-maze—The maze consisted of two open arms (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm, inner diameter) connected by central platform (5 cm × 5 cm) and risen to 40 cm above the floor. The floor of each arm was light grey and the closed arms had transparent (15 cm high) side- and end-walls. The illumination level in all arms was ~150 lx. The mouse was placed in the centre of the maze facing one of the enclosed arms and observed for 5 min. The latency to the first open arm entry, number of open and closed arm entries (four paw criterion) and the time spent in different zones of the maze were measured.

2.2.3. Light–dark exploration—The test was carried out in the open field arena (30 cm × 30 cm, Med Associates, St. Albans, VT) equipped with infrared light sensors detecting horizontal and vertical activity. The dark insert (non-transparent for visible light) was used to divide the arena into two halves, an opening (a door with a width of 5.5 cm and height of 7 cm or a hole with a diameter of 4 cm) in the wall of the insert allowed animal's free movement from one compartment to another. The light half was illuminated by two 40 W light bulb 50 cm above the floor (illumination in the centre of the light compartment ~1000 lx). Animal was placed in the light compartment and allowed to explore the arena for 10 min. Distance travelled, number of rearings, and time spent in different compartments were recorded.

2.2.4. Stress-induced hyperthermia—Stress-induced hyperthermia was assessed by measuring the rectal temperature of the mice twice (rodent thermometer with rectal probe for mice, Bioseb, France). The first measurement served as a baseline measurement and as a stressor at the same time. The second measurement was done 10 min later to reveal the stress-induced hyperthermia reaction.

2.2.5. Spontaneous locomotor activity—The mice were released in the corner of novel open field arena (30 cm × 30 cm, Med Associates). Horizontal and vertical activity was recorded for 1 h (light intensity ~ 150 lx). Peripheral zone was defined as a 6 cm wide corridor along the wall.

2.2.6. Hot plate—Standard hot plate (TSE, Bad Homburg, Germany) was heated to 52 °C and the mouse was confined there by Plexiglas cylinder (diameter 19 cm, height 26 cm). The latency to display licking or shaking of the hindpaw was recorded.

2.2.7. Rotarod—The accelerating rotarod (Ugo Basile, Comerio, Italy) test was performed on two consecutive days. The mice were given three trials a day with an inter-trial interval of 1 h. Acceleration speed from 4 to 40 rpm over a 5-min period was chosen. The latency to fall off was recorded with the cut-off time set at 6 min.

2.2.8. Grip strength—Commercially available grip strength metre (Ugo Basile, Comerio, Italy) was used to measure forelimb grip-strength in mice. The animal was allowed to grasp a bar and pulled by tail. Maximal pulling force (in grams) was recorded when animal lost its grip on the grasping bar. Five trials were performed with inter-trial interval of 1–2 min.

2.2.9. Prepulse inhibition—Mice were enclosed in a transparent plastic tube (Ø 4.5 cm, length 8 cm) that was placed in the startle chamber (Med Associates) with a background white noise of 65 dB and left undisturbed for 5 min. Testing was performed in 12 blocks of 5 trials and five trial types were applied. One trial type was a 40-ms, 120-dB white noise

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acoustic startle stimulus (SS) presented alone. In the remaining four trial types the startle stimulus was preceded by the acoustic prepulse stimulus (PPS). The 20-ms PPS were white noise bursts of 68, 72, 76 and 80 dB. The delay between onset of PPS and SS was 100 ms. The 1st and 12th block consisted of SS-alone trials. In remaining blocks the SS and PPS + SS trials were presented in pseudorandomized order such that each trial type was presented once within a block of 5 trials. The inter-trial interval ranged between 10 and 20 s. The startle response was recorded for 65 ms starting with the onset of the startle stimulus. The maximum startle amplitude recorded during the 65-ms sampling window was used as the dependent variable. The startle response was averaged over 10 trials from blocks 2 to 11 for each trial type. The prepulse inhibition for each PPS was calculated by using the following formula: 100 – [(startle response on PPS + SS trials/startle response on SS trials) × 100].

2.2.10. Fear conditioning—The experiments were carried out employing a computer-controlled fear conditioning system (TSE). Training was performed in a transparent acrylic cage (23 cm × 23 cm × 35 cm) within a constantly illuminated (~100 lx) fear conditioning box. A loudspeaker provided a constant, white background noise (68 dB) for 120 s followed by 10 kHz tone (CS, 76 dB, pulsed 5 Hz) for 30 s. The tone was terminated by a footshock (US, 0.6 mA, 2 s, constant current) delivered through a stainless steel floor grid (Ø 4 mm, distance 10 mm). Two CS-US pairings were separated by a 30 s pause.

Contextual memory was tested 24 h after the training. The animals were returned to the conditioning box and total time of freezing (defined as an absence of any movements for more than 3 s) was measured by infrared light barriers scanned continuously with a frequency of 10 Hz. The CS was not used during this time. Memory for the CS (tone) was tested 2 h later in a novel context. The new context was a similarly sized acrylic box with black non-transparent walls and smooth floor. A layer of wood chips under the floor provided a novel odour to the chamber. After 120 s of free exploration in a novel context the CS was applied for additional 120 s and freezing was measured as above.

2.2.11. Water maze—The system consisted of a black circular swimming pool (Ø 120 cm) and an escape platform (Ø 10 cm) submerged 0.5 cm under the water surface in the centre of one of four imaginary quadrants. The animals were released to swim in random positions facing the wall and the time to reach the escape platform (maximum time 60 s) and the swimming distance were measured in every trial. In addition, thigmotaxis, the time spent swimming within the outermost ring of the pool (10 cm from the wall) was measured. Two training blocks consisting of three trials each were conducted daily. The interval between trials was 4–5 min and between training blocks about 5 h. The hidden platform remained in a constant location for 3 days (6 initial training sessions) and was thereafter moved to the opposite quadrant for 2 days (4 reverse training sessions). The probe trials were conducted approximately 18 h after the last initial and reverse training sessions. The mice were allowed to swim in the maze for 60 s without the platform available. Spatial memory in the probe trials was estimated by preference of swimming in the trained region (imaginary circular area of Ø 30 cm, around the previous platform location) over swimming in corresponding regions in the three other quadrants. After the 2nd probe trial, the mice were tested for one block of 3 trials with the platform made visible in the quadrant not employed previously.

2.2.12. Forced swim test—The mouse was placed for 6 min in the glass cylinder (Ø 18 cm, height 25 cm) filled with water at 23 ± 1 °C to the height of 15 cm. The time of immobility (passive floating, when the animal was motionless or doing only slight movements with tail or one hind limb, whereas the animal was judged to be active when struggling, climbing or swimming using all four paws) was measured in 2 min bins.

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2.2.13. Object exploration—Test was carried out under reduced light conditions (~50 lx) in a large cage (38 cm × 55 cm × 20 cm). The animals were given a 10-min habituation session followed immediately by a 10-min object exploration session. The objects (Object1 – 50 ml Falcon™ tube, height 12 cm, covered with yellow insulation tape; Object2 – Lego™ Duplo™ block, 3 cm × 3 cm × 5 cm) were placed in the centre of arena and time and entries into the object zone were recorded.

2.2.14. Sociability test—The test apparatus consisted of three rectangular compartments (18 cm × 35 cm × 18 cm) divided by Plexiglas walls with openings (6 cm × 5 cm) allowing the animal to move between the compartments [19]. Both side compartments contained an empty transparent Plexiglas holder (8 cm diameter, 10 cm high). The test mouse was first released in the central compartment and was allowed to habituate to the apparatus for 10 min. An unfamiliar sex- and age-matched C57BL/6JOlaHsd mouse (stranger) was placed in one of the holders (with small holes allowing a snout contact between the animals but not biting or other fighting behaviour). The location of the stranger mouse in either of the two holders varied systematically between the trials. The test mouse was then allowed to explore the whole apparatus for 10 min. The time spent in and entries into each compartment were recorded.

2.2.15. Tube test and tube entering—Tube test is commonly used to measure social dominance in mice. Two unfamiliar mice of the same sex but different genotypes were placed in the opposite ends of a 30 cm × 3.8 cm (inner diameter) transparent plastic tube and released simultaneously. The match ended when one mouse completely retreated from the tube. The mouse remaining in tube is designated the winner, and the retreated mouse is the loser. Each animal was tested against all animals from the opposed group. The percent of retreated matches as well as aggressive postures were scored for each animal. Matches lasting more than 2 min or in which animals crossed over each other were not scored.

The same tube was used for assessment of tube entering behaviour with a new cohort of mice. Briefly, the mouse was held by tail and lowered towards the opening of the tube and released when the nose was at the entry. Each mouse was given 5 trials and the number of voluntary entries was recorded. In addition, the time of running through the tube was measured (max. 60 s, if the mouse did not enter the maximum time was assigned).

2.2.16. Resident-intruder test—Resident-intruder test was used to measure social interaction. An intruder mouse (unfamiliar sex- and age-matched animal of C57BL/6JOlaHsd strain) was put in the cage where the test mouse had been acclimatizing for 30 min. Time spent in social activity (sniffing, following, hetero-grooming) and non-social activity (digging, self-grooming and rearing) was recorded during 5 min observation.

2.2.17. Burrowing—Three hours before the beginning of dark period the mice were placed in individual test cages (Type III, 425 mm × 266 mm × 155 mm) containing regular bedding and burrowing tube (a grey plastic tube, 6.3 cm inner diameter, closed from one end, open end was supported by 2 metal bolts, thus raised at 3 cm above the floor) filled with ~255 g of regular food pellets [20]. The amount of food digged out of the tube was measured after 2 and 18 h.

2.2.18. Nest building and grooming—The mice were placed in individual testing cages (Type II, 267 mm × 207 mm × 140 mm) containing regular bedding and 1 h before the dark phase a nestlet of 2.5 g compressed cotton (Ancare, Bellmore, NY) was added into the cage. After 12 h the nests were assessed on a rating scale of 1–5: 1 = Nestlet > 90% intact, 2 = Nestlet 50–90% intact, 3 = Nestlet mostly shredded but no identifiable nest site, 4 =

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identifiable but flat nest, 5 = crater-shaped nest [21]. Grooming behaviour was measured essentially as described in [15].

2.2.19. IntelliCage—Mice were subcutaneously injected with RFID transponders (Datamars SA, Bedano, Switzerland) for individual identification. The IntelliCage (NewBehavior AG, Zurich, Switzerland) is an apparatus designed to fit inside a large cage (610 mm × 435 mm × 215 mm, Tecniplast 2000P) [22]. The apparatus itself provides four recording chambers that fit into the corners of the housing cage. Access into the chambers is provided *via* a tubular antenna (50 mm outer and 30 mm inner diameter) reading the transponder codes. The chamber contains two openings of 13 mm diameter (one on the left, one on the right) which give access to drinking bottles. These openings are crossed by photo beams recording nose-pokes of the mice and the holes can be closed by motorized doors. Four triangular red shelters (Tecniplast, Buguggiate, Italy) were placed in the middle of the IntelliCage and used as sleeping quarters and as a stand to reach the food. The floor was covered with a thick (2–3 cm) layer of bedding. The IntelliCage was controlled by a computer with dedicated software, executing preprogrammed experimental schedules and registering the number and duration of visits to the corner chambers, nose-pokes to the door openings and lickings as behavioural measures for each mouse. In the beginning of the test, the mice were released in the IntelliCage with all doors opened allowing unlimited access to the bottles, and exploratory activity was monitored for 2 days.

The setup for the third cohort was completed by social boxes. A social box consisted of a polycarbonate type III cage (425 mm × 266 mm × 155 mm, with the bedding material on the floor and covered by wire lid) connected *via* a polycarbonate tube (38 cm in length, 4 cm in inner diameter) to the long side of an IntelliCage. On the connecting tube, two antennae register in- and out-ward passages of individual mice [23].

2.3. Statistical analysis

The behavioural data were analyzed using a factorial ANOVA design with genotype, sex and cohort as between-subject factors. If the effect of sex and cohort were not significant the data was pooled. Where appropriate, the model was complemented by within-subject factors to explore the dependence of the main effects on place or time. *Post hoc* analysis after significant ANOVA was carried out by means of Newman–Keuls test. Mann–Whitney *U*-test was used for analysis of non-normally distributed data. Correlational analysis was made by Pearson Product Moment Correlation.

3. Results

No obvious difference in physical appearance (general health, fur, whiskers, body weight) was observed between the Lrrtm1-KO mice and their WT littermates.

3.1. Exploratory and anxiety-like behaviour

3.1.1. Elevated plus maze (Fig. 1a and Table 2)—Lrrtm1-KO mice preferred open arms and avoided entering the closed arms in contrast to their WT littermates (time on open arms: effect of genotype $F(1,83) = 61.0$, $p < 0.0001$; distance on open arms: effect of genotype $F(1,83) = 12.9$, $p = 0.0006$). Importantly, the total distance travelled during 5 min on the elevated plus maze was not different between the groups ($F(1,83) = 0.35$, $p = 0.55$).

3.1.2. Light-dark exploration (Fig. 1b and Table 2)—The Lrrtm1-KO mice did not differ from their WT littermates in latency to enter the dark compartment, in total time spent in the light compartment or in total distance travelled during 10 min in the light–dark arena ($F(1,51) = 0.4$, $p = 0.5$). Moreover, the time spent in the light compartment ($F(1,51) =$

0.0008, $p = 0.98$) and the number of transitions between the compartments ($F(1,51) = 0.34$, $p = 0.56$) were not different between the groups. However, the number of rearings in the light compartment was reduced in the KO mice ($F(1,51) = 11.5$, $p = 0.0013$).

Interestingly, when the size of the opening between the light and dark compartments was reduced by two thirds (from a 38.5 cm^2 hole to a 12.5 cm^2 hole), the latency to enter the dark side was 3.5-times longer in the Lrrtm1-KO mice than in their WT littermates ($F(1,31) = 13.2$, $p = 0.001$). Also, the distance travelled was significantly reduced in the Lrrtm1-KO mice ($F(1,31) = 12.3$, $p = 0.0014$), whereas time spent in the light compartment was prolonged ($F(1,31) = 7.0$, $p = 0.0129$). The KO mice displayed again less rearings ($F(1,31) = 23.5$, $p < 0.0001$).

3.1.3. Object exploration (Table 2)—There was no difference between the genotypes in approaching and exploring the objects, and both groups displayed more interest towards the smaller object (time: effect of genotype $F(1,25) = 0.9$, $p = 0.35$, effect of object $F(1,25) = 5.6$, $p = 0.026$, genotype × object $F(1,25) = 0.2$, $p = 0.69$; frequency: effect of genotype $F(1,25) = 0.7$, $p = 0.40$, effect of object $F(1,25) = 5.8$, $p = 0.023$, genotype × object $F(1,25) = 0.2$, $p = 0.67$).

3.1.4. Stress-induced hyperthermia (Table 2)—The basal temperature and measurement stress-induced hyperthermic reaction were similar between the genotypes (genotype $F(1,25) = 0.06$, $p = 0.8$).

3.1.5. IntelliCage (Fig. 1c–f)—The Lrrtm1-KO mice showed significantly delayed time for the first visit to IntelliCage corner chamber ($p = 0.0022$, *U*-test), for the first nose-poke in the corner ($p < 0.0001$, *U*-test) and for the first visit to the social box ($p = 0.0002$, *U*-test). Reduced exploratory activity was confirmed by lower number of corner visits during initial 6 h in the IntelliCage ($F(1,60) = 35.6$, $p < 0.0001$). During 48 h in the IntelliCage, the Lrrtm1-KO mice made significantly less corner chamber visits ($F(1,60) = 12.7$, $p = 0.0007$), less nose-pokes in the corner chambers ($F(1,60) = 27.7$, $p < 0.0001$) and less visits to social box ($F(1,29) = 31.5$, $p < 0.0001$) than their WT littermates (Fig. 1c). In addition, the average duration of corner chamber visits (Fig. 1d) was shorter in the Lrrtm1-KO mice ($F(1,60) = 27.6$, $p < 0.0001$). Interestingly, we found a significant correlation between the latencies to first transition in light–dark box with small hole and to enter the corner in the IntelliCage ($r = 0.72$, $p < 0.0001$), or to make a nose-poke in the IntelliCage ($r = 0.51$, $p = 0.003$).

3.1.6. Forced swim test (Table 2)—The Lrrtm1-KO mice showed significantly reduced floating during the last 4 min of the test as compared with the WT mice ($F(1,51) = 11.6$, $p = 0.0013$). In contrast, the first episode of immobility appeared earlier (although not significantly) in the Lrrtm1-KO mice ($F(1,51) = 3.9$, $p = 0.055$).

3.2. Social and species-typical behaviour

3.2.1. Sociability test (Fig. 2a)—During the habituation to the test apparatus, the Lrrtm1-KO mice showed a trend towards being less active than their WT littermates (distance: genotype $F(1,51) = 3.4$, $p = 0.07$, phase $F(1,51) = 1.5$, $p = 0.22$, genotype × phase interaction $F(1,51) = 11.0$, $p = 0.0016$) and spent more time in the central compartment of the arena (time: genotype $F(1,51) = 3.8$, $p = 0.058$, side $F(2,102) = 2.4$, $p = 0.092$, genotype × side interaction $F(2,102) = 9.9$, $p = 0.0001$). However, when an unfamiliar mouse (stranger) was placed in one of the side compartments (sociability session), the Lrrtm1-KO mice showed similar preference as the WT mice towards the unfamiliar mouse (genotype $F(1,51) = 0.8$, $p = 0.37$, side $F(2,102) = 17.4$, $p < 0.0001$, genotype × side interaction $F(2,102) = 0.9$, $p = 0.43$).

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3.2.2. Resident-intruder test (Fig. 2b)

The Lrrtm1-KO mice showed significantly more time in social interaction with the intruder mouse ($F(1,31) = 22.0$, $p < 0.0001$).

3.2.3. Tube test (Fig. 2c)

The Lrrtm1-KO mice were more often retreated from the tube ($p = 0.004$, *U*-test). However, during testing we noticed that in several occasions it was difficult to begin the experiment with KO mice, because they did not enter the tube or escaped before direct contact with an opponent. Therefore we decided to test a willingness of mice (from a new cohort) to enter the tube voluntarily.

3.2.4. Tube entering (Fig. 2d)

While all WT mice readily and quickly entered the tube, the Lrrtm1-KO mice showed drastic avoidance of entering the tube ($p < 0.0001$, *U*-test). Typical activity of three WT and four Lrrtm1-KO mice in this test are shown in Supplementary videos.

3.2.5. Burrowing (Table 2)

There was no difference between the genotypes in burrowing either 2 h ($F(1,31) = 2.0$, $p = 0.16$) or 18 h ($F(1,31) = 0.004$, $p = 0.95$) after start of the experiment.

3.2.6. Nest building (Fig. 2e and f) and grooming

The WT mice showed significantly better nest-building abilities than the Lrrtm1-KO mice (material used: $p = 0.0024$; nest score: $p = 0.012$, *U*-test). However, self-grooming behaviour did not differ between the genotypes (Table 2).

3.3. Sensory and motor performance

3.3.1. Spontaneous activity (Fig. 3a)

There was no difference between the genotypes in spontaneous locomotor activity and both groups of mice showed similar habituation as measured by distance travelled over 60 min (effect of genotype $F(1,51) = 1.5$, $p = 0.23$, effect of time $F(11,561) = 35.3$, $p < 0.0001$, genotype × time interaction $F(11,561) = 2.8$, $p = 0.0014$). In addition, no difference was found in time spent in the centre or in the corners of the test arena.

3.3.2. Locomotor stimulation by NMDA-receptor antagonist MK-801 (Fig. 3b)

The Lrrtm1-KO mice displayed weaker locomotor activation after receiving 0.2 mg/kg of MK-801 (effect of genotype $F(1,25) = 9.7$, $p = 0.0045$, effect of time $F(11,275) = 17.1$, $p < 0.0001$, genotype × time interaction $F(11,275) = 2.1$, $p = 0.024$).

3.3.3. Rotarod and grip strength (Table 2)

The Lrrtm1-KO mice exhibited better motor performance than their WT littermates as they stayed longer on the accelerating rotarod (effect of genotype $F(1,51) = 9.4$, $p = 0.0034$). However, grip strength was similar between the genotypes ($F(1,25) = 0.3$, $p = 0.6$).

3.3.4. Hot plate (Table 2)

There was no difference between the groups in latency to nociceptive response on the hot plate ($F(1,51) = 3.2$, $p = 0.078$).

3.3.5. Prepulse inhibition (Fig. 3c and d)

There was no difference between the genotypes in pre-pulse inhibition (effect of genotype $F(1,84) = 0.08$, $p = 0.78$, effect of pre-pulse level $F(3,252) = 33.5$, $p < 0.0001$, genotype by pre-pulse level interaction $F(3,252) = 0.3$, $p = 0.85$). However, the startle response to 120 dB sound stimulus alone was significantly enhanced in the Lrrtm1-KO mice ($F(1,84) = 4.99$, $p = 0.03$).

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3.4. Learning and memory

3.4.1. Fear conditioning (Fig. 4a)—No difference in freezing behaviour (or in distance travelled, data not shown) in any phase of the fear conditioning test was found between the genotypes.

3.4.2. Water maze (Fig. 4b–e)—During the initial training, the Lrrtm1-KO mice displayed reduced average swimming speed (genotype $F(1,24) = 17.7$, $p = 0.0003$) and increased latency to reach the escape platform (genotype $F(1,24) = 8.8$, $p = 0.0067$). However, the distance swum to the platform (genotype $F(1,24) = 1.1$, $p = 0.30$) and amount of immobility (genotype $F(1,24) = 2.2$, $p = 0.15$) were not different from the WT controls. In the first probe trial, the Lrrtm1-KO mice exhibited more immobility than their WT littermates. However, both groups showed a similar preference to the trained location (and time spent near the wall was not increased). The immobility of the KO mice increased further when the platform was moved to the opposite quadrant (genotype $F(1,24) = 14.0$, $p = 0.001$; training session $F(3,72) = 3.95$, $p = 0.012$; interaction of genotype and session $F(3,72) = 3.96$, $p = 0.011$). In the second probe trial, the Lrrtm1-KO mice showed reduced preference to the new location as compared to the WT mice ($F(1,24) = 4.5$, $p = 0.045$).

4. Discussion

The major finding of this study is the exceptional behaviour of Lrrtm1-KO mice to avoid entering small enclosed spaces that was consistently observed in several behavioural tests and in all the three cohorts of mice. Moreover, our extended analysis validated several but not all behavioural findings of a previous study [3] and revealed additional phenotypes in Lrrtm1-KO mice.

4.1. Lrrtm1-KO mice show an extraordinary behaviour of avoiding small enclosures

In the IntelliCage, the mice have to enter the corner chamber through a tube (Ø 3 cm) and then make a nose-poke to an opening (Ø 13 mm) to reach the tip of a water bottle. Lrrtm1-KO mice made less corner chamber visits and nose-pokes, and their latency to the first corner chamber visit and to the first nose-poke was much delayed. This hinted to an apparent aversion to enter small enclosures although other interpretations remained possible. Therefore, we designed additional behavioural tests to test this hypothesis with two additional cohorts of mice.

First, we tested the mice in the light–dark box by changing the size of the opening between the compartments. There was no difference between the genotypes in the latency to escape from the brightly illuminated arena to dark through a standard “large” opening. Under identical light conditions except that the opening was made three times smaller, the escape latency of WT mice remained the same but that of Lrrtm1-KO mice became more than 3-fold longer. The difference between genotypes in latency to enter through the small opening remained when the illumination was further increased or reduced (data not shown). Second, while WT mice readily entered a tube (Ø 3.8 cm) as expected, most Lrrtm1-KO mice did not. Taken together the results indicate that Lrrtm1-KO mice have aversion to enter small spaces.

Going into small spaces, holes or tunnels are a vital species-specific behaviour for small rodents. Interestingly, mice with hippocampal lesions show a phenotype of avoiding entering small holes or tunnels that appears very similar to what we report here in Lrrtm1-KO mice [24]. This suggests that the previously described structural deficits in hippocampal synapses [2,3] may be involved in the circuitry that underlies this aversive phenotype in Lrrtm1-KO mice.

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Of note, although the "avoidance of small enclosures" phenotype was not described in the previous study [3], the prolonged latency of Lrrtm1-KO^{Tak} mice for first head dip in the hole-board test and for entry to the dark compartment in the light–dark box through a tunnel, resemble our findings from the IntelliCage and modified light–dark box, respectively (Table 3). In addition, we suggest that the preference of Lrrtm1-KO mice for open arms in the elevated-plus maze (and for the centre compartment during habituation in the sociability test) may also reflect their aversion to enter small or narrow enclosed spaces.

Conventionally, increased time on the open arms of the plus maze or in the illuminated compartment of the light–dark box is considered to reflect reduced anxiety-like behaviour. However, alternative explanations should be taken into account [25]. As noted above, Lrrtm1-KO mice show specific avoidance behaviour that could be interpreted in terms of face validity for claustrophobia-like behaviour (claustrophobia = fear or aversion of enclosure). Although Lrrtm1-KO mice showed appropriate anxiety, fear and stress responses in other behavioural tests (normal avoidance of light, escape from water, freezing when anticipating shock, and stress-induced hyperthermia) indicating that the mice do not have "generalized anxiety", they showed increased defecation during the light–dark test with small door and slightly increased startle response (that may be related to the mice being enclosed in a tube during the test) suggesting increased anxiety only in the specific test situations when the mice were enclosed or had to enter small spaces.

Phobic disorders, including those related to a specific object or situation, are common among anxiety disorders [26,27]. Interestingly, anxiety disorders are also prevalent in schizophrenia [28] and *LRRTM1* has been previously associated with schizophrenia [13]. To the best of our knowledge, there are currently no animal models for claustrophobia-like behaviour available. Therefore, further behavioural and pharmacological studies are planned to address the underlying mechanism(s) of this novel phenotype in Lrrtm1-KO mice.

4.2. Discrepancies between two independent Lrrtm1-KO mouse lines

Although there are many similarities in the behavioural results between those reported by Takashima et al. [3] and to those reported here, there are a few apparent differences between the two studies (Table 3). In particular, our Lrrtm1-KO mice did not show reduced spontaneous locomotor activity, deficits in spatial memory, or increased freezing as reported in Lrrtm1-KO^{Tak} mice [3]. One possibility is subtle differences in the genetic background of the mice. We compared Lrrtm1-KO mice with their WT littermates (obtained by intercrossing heterozygous mice in C57BL/6JHsd background). The Lrrtm1-KO^{Tak} mice were in C57BL/6J background but whether WT littermates were used as controls was not described [3]. Another possibility is methodological differences and/or environmental factors. For example, differences in housing conditions between the studies (Table 3) could explain the difference in spontaneous locomotor activity [29]. On the other hand, locomotor activity induced by MK801 using a dose of 0.5 mg/kg was not different between the genotypes (Fig. 7A in [3]), whereas MK801 dose of 0.2 mg/kg as used here revealed a significant difference between the genotypes, possibly because 0.2 mg/kg of MK801 appears to stimulate locomotor activity more than 0.5 mg/kg that already induces stereotypic behaviour [30,31].

In the water maze test, both Lrrtm1-KO strains demonstrated normal spatial learning of the hidden (and visible) platform. The Lrrtm1-KO^{Tak} mice showed reduced crossings of target in the (first) probe test suggesting impaired spatial memory [3]. In contrast, our Lrrtm1-KO mice were not impaired in searching for the target in the first probe trial. During the reversal learning, our KO mice showed substantially reduced swim speed and increased immobility. Thus, although our Lrrtm1-KO mice were impaired in the second probe trial, is not obvious that the result reflects impaired spatial memory or rather a stress-related response. Overall,

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the water maze results suggest reduced behavioural flexibility and adaptation in Lrrtm1-KO mice consistent with "altered behavioural responses to novel environments and stressful situations" as proposed [3].

Finally, although Lrrtm1-KO^{Tak} mice showed increased freezing already before the fear conditioning test, the difference in freezing remained the same during the test. This is consistent with our Lrrtm1-KO mice that did not differ from WT controls in the fear conditioning test suggesting that lack of LRRTM1 does not impair fear learning and memory.

4.3. LRRTM1-deficiency may be associated with thalamocortical hypofunction

Lrrtm1 mRNA is predominantly expressed in thalamus, hippocampus and limbic cortex including neurons in the retrosplenial cortex that are selectively vulnerable to NMDA-receptor antagonist MK801 toxicity [13] (see Suppl. Fig. 1). The behavioural and toxic effects of MK801 are thought to be mediated *via* disinhibition of excitatory thalamocortical networks [32–34]. Given the reported morphological defects in hippocampal synapses [2] and possibly also in somatosensory cortex [3], we hypothesize that the in Lrrtm1-KO mice may have impaired function of excitatory synapses also in the thalamocortical network. Consistent with this idea, locomotor activity in Lrrtm1-KO mice was significantly less stimulated by low dose of MK801. Whether a thalamocortical hypofunction manifests at the synapse level in the Lrrtm1-KO mice remains to be studied.

4.4. Lrrtm1- and Nrxn1a-KO mice exhibit some similarities in behaviour

Given the reported interaction of LRRTM1 and neurexin-1 it seems relevant to compare the behavioural phenotypes in Lrrtm1-KO mice to those reported in mice lacking neurexin-1a [15]. One obvious difference between the Lrrtm1-KO and Nrxn1a-KO mice is in the self-grooming behaviour (a measure of repetitive behaviour). Since the grooming behaviour was normal in Lrrtm1-KO mice, the increased repetitive behaviour in Nrxn1a-KO mice presumably reflects neurexin-1 binding to neuroligin-1 [35]. However, there are also several similarities: (i) Both Lrrtm1- and Nrxn1a-KO mice show normal spontaneous locomotor activity but remain longer on the rotarod than their WT controls. (ii) Both Lrrtm1- and Nrxn1a-KO mice exhibit reduced nest building. (iii) Both Lrrtm1- and the Nrxn1a-KO mice show increased immobility and slower swim speed in the water maze but apparently normal water maze learning and memory. (iv) In the elevated plus maze, Nrxn1a-KO mice showed only a trend of increased time in the open arm, while Lrrtm1-KO mice stay clearly longer in the open arm. Whether Nrxn1a-KO mice exhibit a similar avoidance of enclosure as reported here in the Lrrtm1-KO mice remains to be studied. Consistent with the idea that LRRTM1 may be a major binding partner for NRXN1a *in vivo*, the expression patterns of *Lrrtm1* and *Nrxn1* mRNAs in postnatal mouse brain appear similar (see Suppl. Fig. 1). LRRTM1 function *in vivo* is likely to be compensated by LRRTM2 since their expression in brain [1], binding to NRXN1 [5,6] and activity *in vitro* [8,9] is overlapping.

5. Conclusion

In sum, our extended behavioural analysis of the Lrrtm1-KO mice has revealed an unusual phenotype expressed as aversion to enter small enclosures. Our results validate part of the behavioural data of another Lrrtm1-KO mouse line, but indicate that abnormal results from behavioural tests in which the mice have to enter a small door or tunnel may reflect this extraordinary phenotype. Further, our comparative analysis with Nrxn1a-KO mice phenotypes highlights the potential *in vivo* functions of the reported interaction of LRRTM1 and neurexin-1.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- The LRRTM family proteins are synaptogenic cell adhesion molecules and are associated with neuropsychiatric disorders.
- LRRTM1-knockout mice show specific avoidance of entering narrow space.
- This phenotype was confirmed by several different tests in a comprehensive behavioural screen.
- We propose LRRTM1-knockout mice as a tentative model of claustrophobia-like behaviour.

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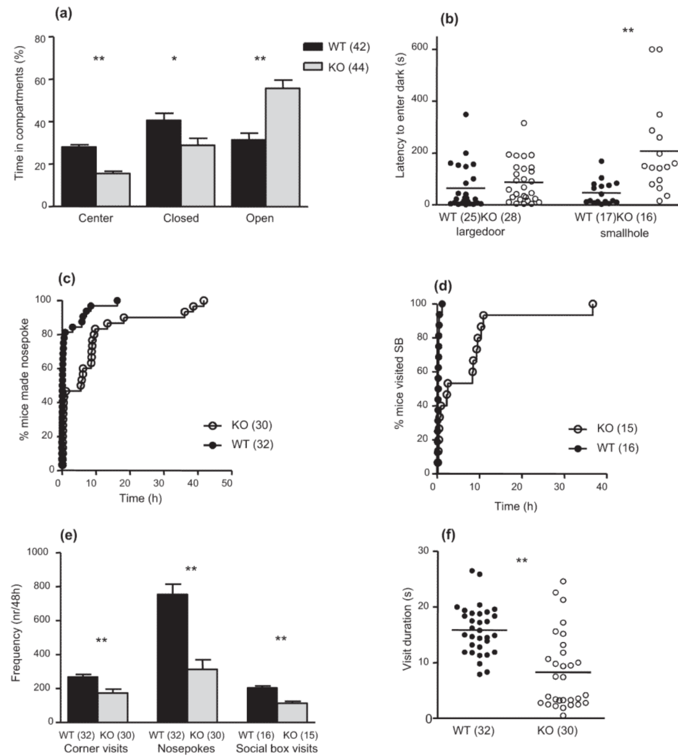

Fig. 1.

Abnormal behaviour in Lrrtm1-KO mice in several tests is consistent with aversion to small enclosures. (a) In the elevated plus maze, Lrrtm1-KO mice spend more time in the open arms in contrast to their WT littermates. (b) In the light–dark box, escape time from the light (1000 lx) compartment through a "large" opening (38.5-cm^2) was similar between the genotypes (left). In contrast, escape time from the light compartment through a "small" opening (12.5-cm^2) was much longer in Lrrtm1-KO mice than in their WT littermates (right). (c and d) In the IntelliCage, latency to make the first nose-poke in the corner chamber (c) and first visit to the social box is longer in the Lrrtm1-KO mice than in their WT littermates (d). (e) Lrrtm1-KO mice visit the corner chambers less often and make less nose-pokes there than their WT littermates. Also, the number of visits to external social box is reduced in Lrrtm1-KO mice. (f) The average duration of individual corner chamber visits made by Lrrtm1-KO mice is shorter than those made by their WT littermates (horizontal lines mark the mean values). Data represents mean ± SEM. *$p < 0.05$; **$p < 0.01$ compared to WT. Number of mice in each test is shown in parenthesis.

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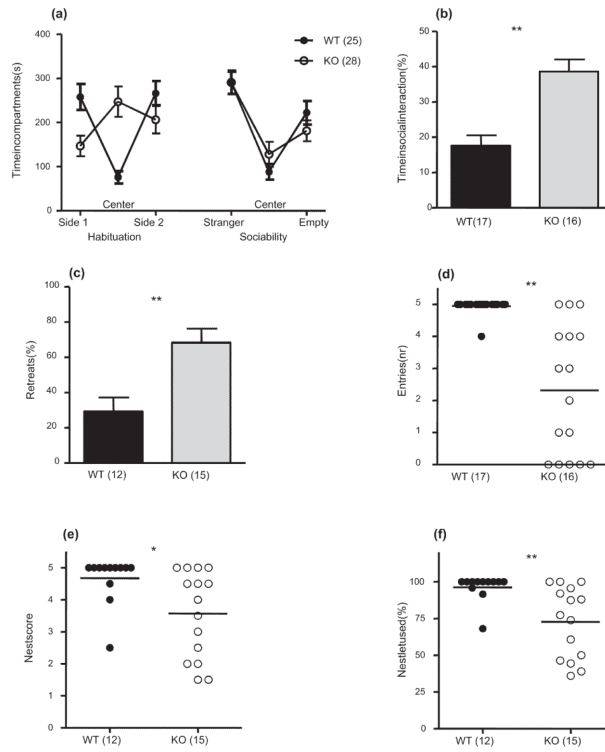

Fig. 2.

Lrrtm1-KO mice exhibit alterations in social interaction and impaired nest building. (a) Time spent in different compartments during the sociability test. Lrrtm1-KO mice spent more time in the centre compartment during the habituation as if they avoided entering through a hole to the side compartments. (b) Lrrtm1-KO mice displayed significantly enhanced time in social interaction during resident-intruder test. (c) Lrrtm1-KO mice appeared to retreat more often in the tube test (cohort-2). (d) Lrrtm1-KO mice avoided entering the tube (cohort-3). (e and f) Lrrtm1-KO are impaired in nest building: nest score (e) and amount nest material shred (f) is reduced. Horizontal lines mark the mean values. Data represents mean ± SEM. $*p < 0.05$; $**p < 0.01$ compared to WT. Number of mice in each test is shown in parenthesis.

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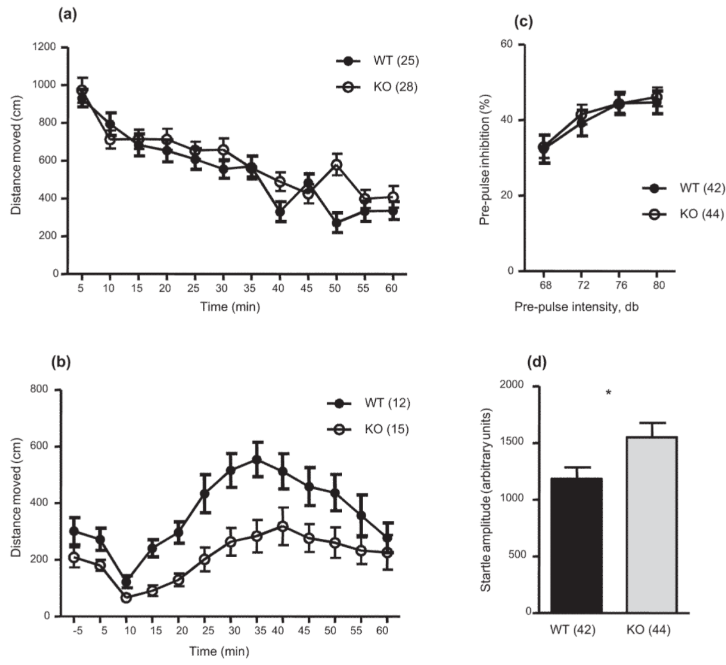

Fig. 3.

Normal spontaneous activity, but reduced MK-801-induced locomotion and enhanced startle response in Lrrtm1-KO mice. (a) Spontaneous locomotor activity in a novel test arena was similar between the genotypes during a 60 min observation. (b) Administration of MK-801 (0.2 mg/kg) induced less locomotor activity in the Lrrtm1-KO mice than in their WT littermates. (c) Prepulse inhibition of auditory startle response was not affected in Lrrtm1-KO mice. (d) Startle reflex was increased in Lrrtm1-KO mice. Data represents mean ± SEM. *$p < 0.05$ compared to WT. Number of mice in each test is shown in parenthesis.

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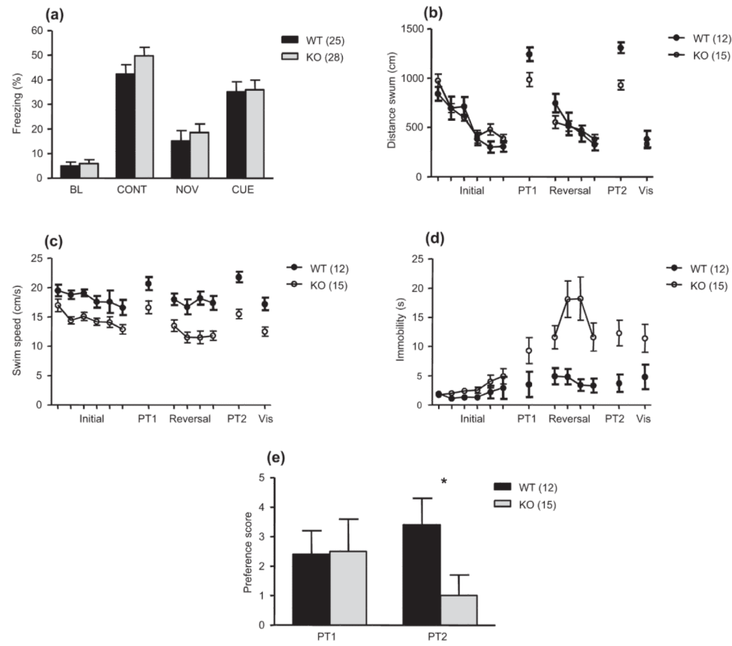

Fig. 4.

Deletion of *Lrrtm1* does not impair learning and memory. (a) The percentage of freezing was similar between the genotypes in all phases of the fear conditioning procedure. BL = baseline freezing before training, CONT = freezing 24 h after the training (contextual memory), NOV = freezing response to a novel context before the cue, and CUE = freezing response to cue (tone) in the novel context. (b–e) Morris water maze task. (b) Lrrtm1-KO mice and their WT littermates swam equal distances to find the hidden and reversed platforms. However, the Lrrtm1-KO mice exhibited slower swimming speed (c) and progressively more immobility (d) during the water maze task. (e) Lrrtm1-KO mice preferred the previously trained platform location as much as their WT littermates in the 1st probe trial but less than the WT mice in the 2nd probe trial. Preference to the trained platform region in probe trials was calculated as the number of crossings of the trained region minus average number of crossings of the corresponding three other regions. Data represents mean ± SEM. *$p < 0.05$ compared to WT. Number of mice in each test is shown in parenthesis.

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Table 1

Behavioural analysis of Lrrtm1-KO mice: cohorts and tests applied

Cohort	Tests	Animals	
		WT	KO
Cohort-1	EPM; LD; OF; HP; RR; PPI; FC; SOC; FST; IC	10 F + 3 M	7 F + 6 M
Cohort-2	EPM; LD; OF; HP; RR; PPI; FC; WM; SOC; SD; OBJ; FST; GRIP; GROOM; NEST; SIH; MK-801; IC	6 F + 6 M	8 F + 7 M
Cohort-3	EPM; LDs; PPI; BURROW; TUBE; RI; IC +SB	9 F + 8 M	9 F + 7 M

Abbreviations: WT, wild type; KO, knock-out; F, females; M, males; EPM, elevated plus maze; LD, light–dark box; OF, open field; HP, hot plate; RR, rota-rod; PPI, pre-pulse inhibition; FC, fear conditioning; SOC, sociability test; FST, forced swim test; IC, IntelliCage; WM, water maze; SD, social dominance (tube test); OBJ, object exploration; GRIP, grip strength; GROOM, grooming; NEST, nest-building; SIH, stress-induced hyperthermia; MK-801, drug-induced activity in open field; LDs, light–dark box with small door; BURROW, burrowing test; TUBE, tube entering; RI, resident-intruder test; SB, social box.

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Table 2

Behavioural analysis of Lrrtm1-KO mice: additional results.

	Mean ± SEM		*p*-Value
	WT	KO	
Body weight (9-wk-old)			
Males	25.6 ± 0.5	24.7 ± 0.3	0.099
Females	18.6 ± 0.2	18.9 ± 0.2	0.81
Elevated plus maze			
Faecal boli (nr)	0.7 ± 0.2	0.3 ± 0.1	0.052
Distance (cm)	1320 ± 50	1270 ± 50	0.56
Distance centre (%)	22.1 ± 0.6	15.5 ± 0.7	0.0001
Distance closed (%)	45 ± 3	32 ± 3	0.0032
Distance, open (%)	33 ± 3	52 ± 3	0.0000
Latency to open entry (s)	24 ± 7	20 ± 4	0.58
Light–dark box with large door			
Faecal boli (nr)	1.0 ± 0.3	0.9 ± 0.3	0.68
Distance (cm)	1700 ± 80	1780 ± 90	0.52
Latency to dark entry (s)	64 ± 17	87 ± 15	0.32
Time in light (%)	39 ± 4	39 ± 3	0.98
Rearings (nr)	71 ± 6	49 ± 5	0.011
Transitions (nr)	46 ± 3	44 ± 3	0.56
Light–dark box with small door			
Faecal boli (nr)	0.2 ± 0.1	1.3 ± 0.5	0.028
Distance (cm)	1640 ± 100	1160 ± 90	0.0014
Latency to dark entry (s)	46 ± 11	210 ± 40	0.0010
Time in light (%)	34 ± 4	53 ± 7	0.013
Rearings (nr)	85 ± 11	25 ± 5	0.0000
Transitions (nr)	19 ± 1	15 ± 2	0.10
Forced swim test			
Latency to immobility (s)	67 ± 5	51 ± 6	0.055
Immobility (%)	65 ± 2	54 ± 3	0.0013
Spontaneous activity			
Distance (cm)	6500 ± 400	7300 ± 400	0.23
Time in centre (%)	13.2 ± 1.5	14.9 ± 1.1	0.39
Time in corners (%)	65 ± 3	63 ± 2	0.49
Hot plate			
Latency (s)	16.6 ± 0.7	15.0 ± 0.6	0.078
Rota-rod			
Latency to fall (s)	259.1 ± 9.7	298.4 ± 8.4	0.0034
Grip strength			
Pulling force (g)	57 ± 2	55 ± 3	0.60
Stress-induced hyperthermia			

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	Mean ± SEM		*p*-Value
	WT	**KO**	
Basal temperature (°C)	36.5 ± 0.1	36.3 ± 0.2	0.4817
Increase (°C)	0.9 ± 0.1	1.0 ± 0.1	0.3474
Object-exploration			
Object1, time	8 ± 4	23 ± 10	0.23
Object1, frequency	5.6 ± 2.0	8.5 ± 1.4	0.24
Object2, time	28 ± 18	50 ± 21	0.44
Object2, frequency	9 ± 3	11 ± 2	0.63
Grooming			
Duration (s)	41 ± 8	46 ± 13	0.76
Frequency (nr)	7.0 ± 0.7	8.6 ± 2.0	0.52

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Table 3

Comparison of behavioural phenotypes of Lrrtm1-KO^{Air} and Lrrtm1-KO^{Tak} mice .[a]

	Lrrtm1-KO^{Air}	**Lrrtm1-KO^{Tak}**
Genetic background	C57BL/6JOlaHsd	C57BL/6(?)
Littermate controls	Yes	Unclear
Sex	Males and females	Males
Housing	Group housed	Single housed
Open field activity		
70 lx	ND	No change
150 lx	No change	ND
250 lx	ND	Decreased
Elevated plus maze		
Distance	No change	No change
Time on open arms	Increased	Increased
Light–dark box		
Latency to dark	Increased (hole Ø 4 cm); no change (door 5.5 cm × 7 cm)	Increased (tunnel 3 cm × 5 cm)
Hole-board		
Head-dip latency	ND	Increased
IntelliCage		
Nosepoke latency	Increased	ND
Water maze		
Immobility	Increased	No change
Probe trial target preference	No change	Decreased
Fear conditioning		
Freezing before conditioning	No change	Increased
Freezing to context	No change	Increased
Freezing to cue	No change	Increased
Rotarod		
Time stayed on rotating rod	Increased	No change
Hot plate		
Latency licking hindpaws	No change	No change
Forced swim test		
Immobility	Decreased	No change
Startle response	Increased	No change
Pre-pulse inhibition	No change	No change

[a]Selected tests performed in both studies. ND, not done. Lrrtm1-KO^{Air} (this study), Lrrttm1-KO^{Tak} [3].