Published in final edited form as:

Behav Brain Res. 2011 November 20; 225(1): 142–150. doi:10.1016/j.bbr.2011.07.008.

Are Sema5a mutant mice a good model of autism? A behavioral analysis of sensory systems, emotionality and cognition

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Abstract

Semaphorin 5A (Sema5A) expression is reduced in the brain of individuals with autism, thus mice with reduced Sema5A levels may serve as a model of this neurodevelopmental disorder. We tested male and female Sema5a knockout mice (B6.129P2SEMA5A^{<TM1DGEN>}/J) and C57BL/6J controls for emotionality, visual ability, prepulse inhibition, motor learning and cognition. Overall, there were only two genotype differences in emotionality: Sema5a mutant mice had more stretch-attend postures in the elevated plus-maze and more defecations in the open field. All mice could see, but Sema5a mice had better visual ability than C57BL/6J mice. There were no genotype differences in sensory-motor gating. Sema5a mice showed higher levels of activity in the elevated plus-maze and light/dark transition box, and there were sex by genotype differences in the Rotarod, suggesting a sex difference in balance and coordination differentially affected by Sema5a. There were no genotype effects on cognition: Sema5a mice did not differ from C57BL/6J in the Morris water maze, set-shifting or cued and contextual fear conditioning. In the social recognition test, all mice preferred social stimuli, but there was no preference for social novelty, thus the Sema5A mice do not have a deficit in social behavior. Overall, there were a number of sex differences, with females showing greater activity and males performing better in tests of spatial learning and memory, but no deficits in the behavior of Sema5A mice. We conclude that the Sema5a mice do not meet the behavioral criteria for a mouse model of autism.

Keywords

semaphorin 5A; autism; mouse; cognition; anxiety

1. Introduction

The semaphorins are a class of proteins which share a conserved domain of 500 amino acids and function to guide axon growth cones during neural development [1]. Class 5 semaphorins have seven thrombospondin type-1 repeats, and function to promote neurite outgrowth [2, 3]. Semaphorin 5A (Sema5A) is an integral membrane-bound protein that binds to plexin-B3, heparan sulfate proteoglycans (HSPGs), chondroitin sulfate

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proteoglycans (CSPGs) or Syn-3 and functions in cell morphology, cytoskeletal organization, neural connectivity and vasculature patterning [4]. *Sema5A* can act as an attractant or repellant for axons during development, depending on the location of its expression and the presence of binding proteins. In the visual system, *Sema5A* is expressed in the optic disc and along the optic nerve, inhibiting retinal axon bundles from straying from the main axon bundle of the optic nerve [2]. *Sema5a* plays an essential role in embryonic development, as homozygous *Sema5a* knockout mice on a 129/Sv/NMRI background died between embryonic days 11.5 and 12.5, possibly due to impaired growth and development of cranial blood vessels [3]. Heterozygous *Sema5a* mice were healthy and viable, despite a reduced level of *Sema5A* protein, and there were no differences between heterozygous and wildtype mice in blood vessel or neural development up to embryonic day 12.5 [3]. Homozygous *Sema5a* mice were not found to be embryonic lethal on a C57BL/6/129/OlaHsd background [5]. This may be due to compensatory mechanisms in the background C57BL/6 or 129/OlaHsd mice, or flanking gene effects from the 129/OlaHsd embryonic stem cells [6, 7], similar to the Alzheimer's model mouse Tg2576 transgene being lethal on an FVB/N or FVB/N cross background, but viable on a C57BL/6 and SJL background [8].

A genome-wide association study found a single nucleotide polymorphism (SNP) on human chromosome 5p15 between *SEMA5A* and *TAS2R1* which was associated with autism, a pervasive neurodevelopmental disorder characterized by reduced social behavior and stereotypy, as well as reduced expression of *SEMA5A* in the brains of autistic patients [9], suggesting a role for *Sema5A* in neural development and neurological disease. The core symptoms of autism are generally categorized as impairments in social interactions and social communication, resistance to change and repetitive behaviors or stereotypies, with secondary features such as anxiety, mental retardation, clumsiness, sleep disturbances, and seizures [10–13]. Deficits in sensorimotor gating, as measured by prepulse inhibition, have also been found [14, 15].

While it is difficult to model a multifaceted disease such as autism in mice, there are certain behaviors in mice that are analogous to those found in the autism spectrum disorders, and these have been used to determine the validity of various genetic and inbred mouse models of autism [16–26]. A mouse model of autism should replicate at least one core symptom of the human disorder, if not all three, and some of the secondary symptoms [16].

The purpose of this experiment, therefore, was to assess core autistic-like behaviors in *Sema5a* mutant mice on a mixed B6/129P2 background, as well as the associated symptoms, which form the behavioral phenotype of autism spectrum disorders (see Table 1). A phenotypic screen did not find differences in the open field, tail suspension test, Rotarod, prepulse inhibition, tail flick test, hot plate test between homozygous and wildtype control males [5]. Our behavioral test battery measured social behavior (social preference and social novelty preference), motor coordination and motor learning (Rotarod), hyper/hypoactivity (open field), repetitive behavior (open field), resistance to change (set shifting, reversal learning in the Morris water maze), anxiety (elevated plus-maze, light/dark box), idiosyncratic responses to sensory stimuli (visual water box, prepulse inhibition) and learning and memory impairments (Morris water maze, cued and contextual fear conditioning). We hypothesized that *Sema5a* mice would exhibit significantly more “autism-like” behaviors than the C57BL/6J controls.

2. Methods

2.1 Mice

Ten male and 10 female B6.129P2-*Sema5a*^{<Tm1Dgen>/J} mice (*Sema5a* mutant mice; JAX # 005834) and 10 male and 10 female C57BL/6J mice (wildtype; JAX # 000664) were purchased from Jackson Laboratories (Bar Harbor, ME). The *Sema5a* mutant mice had a targeted insertion mutation, in which bacterial lacZ gene was inserted into the *Sema5a* gene such that the endogenous gene promoter drove expression of beta-galactosidase [5]. The *Sema5a* mice were genotyped prior to shipping by Jackson Laboratories and all mice carried the Deltagen allele. Beta-galactosidase staining by Jackson Laboratories demonstrated good staining in all organs, including the brain, suggesting beta-galactosidase was being expressed from the endogenous *Sema5a* promoter as expected. As the *Sema5a* mutant mice had been backcrossed to C57BL/6J mice for at least seven generations, C57BL/6J was the appropriate wildtype control mouse.

Mice arrived in our lab at 12 weeks of age and were housed in same sex, mixed genotype groups of four in clear, plastic cages (18.75 × 28 × 12.5 cm). The housing cages had stainless steel food hoppers and wood shavings were used as bedding. Mice were fed Purina rodent chow (#5001) and tap water ad libitum (except where mentioned). The housing room was maintained at a temperature of 22 ± 2° C and on a reversed 12:12h light:dark cycle (lights off at 0930). Mice were individually identified using an ear punching code.

Mice were tested during their active (dark) phase of their light/dark cycle and were 5–6 months of age when testing began and 9–10 months of age when testing was completed. Body weight was analyzed when mice were tested on the Rotarod. Ten males (five *Sema5a* mice and five C57BL/6J mice) were individually housed due to fighting when housed in groups. As separation from group housing has been shown to cause depressive-like behaviors in mice [27], separate analyses were performed comparing group and separated males for each measure, and significant results are reported. Mice were tested in the order given below in a battery of ten tests, which were designed to detect autistic-like behavior in mice. The experimenter was blind to genotype as mice were numbered without reference to genotype.

2.2. The Elevated Plus-Maze (EPM)

The EPM had two open arms (30 × 5 cm) with a slight ledge (4 mm) to prevent mice from falling, and two closed arms (30 × 5 × 15 cm high walls) radiating from a 5 × 5 cm central square [28]. The floor was made of black Plexiglas and the walls of clear Plexiglas. The apparatus was located in a laboratory room (2 × 5 m) and lit by a 60-Watt red lamp. Mice were placed in the center square of the EPM facing an open arm and their behavior was scored for 5-minutes, after which they were removed from the maze and returned to a holding cage. The number of defecations and urinations were recorded and then the maze was cleaned with a solution of 70% ethyl alcohol to eliminate olfactory cues, and permitted to dry before the next mouse was tested. Each trial was recorded using a video camera-based computer tracking system (Limelight, Actimetrics) on an IBM PC computer with the camera fixed to the ceiling 2.1 m above the apparatus. The tracking system recorded time spent in each arm, while the experimenter scored line crosses, rearing, stretch-attend postures, head dips, grooming and freezing live using the Limelight computer program. This task measured anxiety and locomotor activity.

2.3. Light/Dark Transition Box

The light/dark box (45 × 27 × 27 cm) was made of plywood and consisted of two compartments of unequal size as described by Costall et al [29]. The small compartment (18

$\times 27$ cm) was painted black (2/5 of the box) and the larger compartment (27×27 cm) was painted white (3/5 of the box). They were connected by a floor level door (7.5×7.5 cm) in the center of the wall between the two compartments. The floor was covered in Plexiglas and both compartments were covered with lids of clear Plexiglas. A 60-Watt white light located 40 cm above the center of the white compartment provided bright illumination. The apparatus was located in a 2×5 m laboratory room. Mice were placed in the center of the white compartment facing the door and allowed to explore the apparatus for 5 minutes. The experimenter scored the behavior live using Hindsight for MS-Dos Version 1.5 computer software and a video camcorder located 150 cm above the center of the maze recorded the behavior. The mice were then removed from the apparatus and returned to a holding cage. The number of defecations and urinations were recorded, and the maze was cleaned with a solution of 70% of ethyl alcohol and permitted to dry between tests. The time spent in the light and dark zones, number of transitions between zones, number of rears, number of stretch-attend postures, grooming duration and freezing duration were recorded. This task measured anxiety and locomotor activity.

2.4. Open Field

The open field was constructed of plywood (72×72 cm) with 36 cm high walls. The walls and floor were both painted white. Lines were drawn on the floor to divide it into sixteen 18×18 cm squares. A central square of equal size was drawn in the middle of the open field [28] and the floor was covered with Plexiglas. The open field was located in a 2×5 m test room which was lit by two 60-watt red lamps. Mice were placed randomly into one of the four corners of the open field and were allowed to explore the apparatus for 5-minutes while the experimenter scored the behaviors using the Limelight program. Each trial was recorded using a video camera-based computer tracking system (Limelight, Actimetrics) on an IBM PC computer with the camera fixed to the ceiling, 2.1 m above the apparatus. The experimenter scored time in the centre square, rearing, stretch-attend postures, grooming and freezing. The mice were returned to their home cages and the number of defecations and urinations recorded, after which the open field was cleaned with 70 % ethyl alcohol and permitted to dry between trials. To assess habituation to the novelty of the arena, mice were exposed to the apparatus for a second 5-minute test 24 hours later [30, 31]. Habituation was measured using an activity change ratio [Day 2 line crosses/(Day 1 line crosses + Day 2 line crosses)] to control for any baseline activity differences between the genotypes [32]. This task measured anxiety, locomotor activity and habituation.

2.5. Acoustic Startle & Prepulse Inhibition

Acoustic startle and prepulse inhibition (PPI) of startle were measured in the SR-Lab System (San Diego Instruments, San Diego, CA, U.S.A.). Mice were placed into a restraining cylinder (12.8×3.5 cm internal diameter or 12.8×3.3 cm internal diameter, depending on the size of the mouse), and the cylinder was mounted on a 12.8×20.3 cm platform containing a piezoelectric accelerometer, which measured the movements produced by the mouse. The cylinder was placed in a $38.1 \times 40.6 \times 58.4$ cm sound-attenuated cabinet, which contained a high-frequency loudspeaker. The vibrations of the cylinder produced by the movements of the mouse were converted into a digital signal recorded by a desktop computer (PC), via an analog-to-digital relay. Mice were placed into the cylinder and allowed to acclimatize for 5 minutes with the background noise set at 70 dB. This was followed by 6 startle trials (120 dB), and 5 blocks of 7 different trial types (no stimulus; prepulse 74, 78, 82, 86 or 90; startle 120 dB). The ITI for the session was 10 to 20s, with an average of 15s. The total time period for the session was approximately 16 minutes per mouse. The % PPI of the acoustic startle response (ASR) was calculated by: $100 - [(V_{\max} \text{ of PPI trials} / V_{\max} \text{ of startle alone trials}) \times 100]$ [33]. This task measured hearing and sensorimotor gating.

2.6. Rotarod

Motor coordination and motor learning were measured using the AccuRotor Rotarod (Accuscan Instruments Inc. Columbus, Ohio), which consists of a rotating acrylic rod (3-cm diameter), divided into four 11-cm wide sections by Plexiglas circular dividers (15-cm high). Mice were weighed each day and then carried in their home cages to the testing room and tested in squads of four, with one mouse in each section of the rod. Four automatic timers started timing the moment the rod was set into motion and turned off automatically when the mice fell into the holding chamber 39 cm below the rod. The Rotarod was located in a small room (1.12 × 2.60 m) which was illuminated with a single 60-Watt red light bulb. The Rotarod was set to accelerate from 0.0 rpm to 48.0 rpm over a 6-minute trial (acceleration 8 rpm/min). At the beginning of each trial, mice were placed on the rod facing the opposite direction to the rotation and the Rotarod was turned on. After each mouse fell into the chamber, it was left there until all mice fell, after which there was a 1-minute inter-trial interval before the next trial began. If a mouse was able to stay on the rod for the full 6-minutes, the motor was turned off and the mouse was placed in the chamber below for the 1-min rest period. The apparatus was cleaned with soap and water and wiped dry with paper towel after each group of mice were tested. A total of 42 trials were completed for each mouse with 6 trials per day over 7 days of testing [34].

2.7. Visual Detection in the Visual Water Box

The visual testing apparatus consisted of a trapezoidal-shaped pool (140 cm long × 80 cm wide at one end and 25 cm wide at the other) and was made of 6-mm clear Plexiglas with 55-cm (high) walls and painted black. It was placed on a solid table (146-cm long × 100-cm wide × 46-cm high) and two computer-controlled monitors were placed side by side outside the wide end [as described by 35]. A midline divider (41-cm long and 40-cm high) painted black was placed in the pool between the computer screens. A release alley (35-cm long × 7-cm wide × 20-cm high) centered at the narrow end of the pool was also painted black. A movable escape platform (37-cm long × 13-cm wide × 14-cm high) made of clear Plexiglas was placed below the positive visual stimulus computer screen. The pool was filled with water (22°C) to a depth of 15-cm. Screen reflections from the visual stimuli on the surface of the water made the platform invisible from water level. Mice were tested in two phases: shaping and visual detection.

On the pre-training day mice were shaped to locate the hidden platform below a screen displaying a vertical grating (0.17c/deg) on one side of the divider while a grey screen was shown on the other side of the divider with no platform below it. The positive stimulus (S+) was alternated between the left and right side of the divider according to the sequence described by Wong and Brown [35]. Mice were placed into individual holding cages lined with paper towel to dry them between trials. Each mouse was removed from the holding cage with a plastic bucket and transferred into the apparatus. Shaping trials involved placing the mice on the platform on each side of the divider and then releasing them in the pool at the divider line on each side and finally from the starting chute.

The visual detection task requires the mouse to differentiate between a vertical grating of 0.17 cycles per degree (c/deg or CPD) and a grey screen. The vertical grating was the S+ and indicated the presence of the hidden platform. The grey screen was the negative stimulus (S-). To score a correct trial mice must swim directly to the S+ within 60-seconds. An error was scored if the mouse passed the S- threshold, which was the end of the divider between the two screens, or took more than 60-sec to find the S+ platform. If a mouse made an error, it was led to the platform using the plastic bucket, then removed from the platform and placed in the release chute and immediately required to run another trial (error trial). The mice were trained for 8 trials a day for 8 days (64 trials). Criterion for visual detection

was met when the mouse reached 70% correct over the 8 test trials. After each trial mice were removed from the platform with the bucket and placed into the holding cage to dry. The ITI was 5 minutes. This task measured visual ability.

2.8. Morris Water Maze

The Morris water maze was constructed from a black circular polypropylene pool measuring 110-cm in diameter and 20-cm deep. The pool was filled to a depth of 14-cm with room-temperature ($22 \pm 1^\circ\text{C}$) tap water, which was made opaque with the addition of 100-mL of non-toxic white liquid tempera paint (Schola, Marieville). A Plexiglas cylinder ($13.75 \text{ cm} \times 9 \text{ cm}$ diameter) was used as the escape platform. This platform had a removable red and yellow striped top ($3 \text{ cm} \times 9 \text{ cm}$ in diameter) with a colorful flag erected in the center. For visible platform tests the level of the water in the pool was adjusted to 0.5-cm below the surface of the striped top, creating a visible escape platform, or to 0.5-cm above the white cylinder (with the striped top removed), creating a hidden escape platform. The pool was located in a testing room ($5.2 \times 2.4 \text{ m}$) which had posters on the walls, cupboards and furniture (table, chairs) which served as visual cues. During testing, the room was dimly lit with diffuse white light (30 lux). The performance of the animals in the water maze was recorded using a video camera-based computer tracking system (Watermaze, Actimetrics) on an IBM PC computer, with the camera fixed to the ceiling 2.1 m above the pool.

Acquisition (3 days) and reversal training (3 days) was conducted with the hidden platform. During acquisition the platform was in the southwest quadrant, and during reversal the platform was moved to the opposite side of the maze. Each mouse completed 4 trials per day for 12 trials of acquisition training and 12 trials reversal training, each trial from a different one of the 4 start locations [34]. Mice were run in squads of 4–6 with 5-minute inter-trial intervals. In each trial, the mouse was given a maximum of 60-sec to locate the escape platform. When the mouse located the platform, the timer was stopped and it was allowed to stay on the platform for 10-sec, after which it was returned to the holding cage which contained paper toweling to dry the mouse. If the mouse did not find the platform during the allotted time, it was guided onto the platform using the plastic container. During the 60-second probe trial (day 7) no escape platform was present so that visuo-spatial memory could be assessed. On the visible-platform day (day 8) the platform was moved to another quadrant of the pool and the visible top was added to the platform to assess visual ability and motivation to locate the platform. This task measured visuospatial learning and memory.

2.9. Set Shifting

Mice were food restricted to 85 - 90% of their free-feeding weight for one week before testing. Mice were weighed in the morning and fed in the afternoons, with the amount of food given determined by how much weight was lost by each mouse. Food pellets were broken into small (~ 1g) pieces and scattered around the cage for group-housed mice to ensure that all mice obtained food and mice were given small pieces of sugar for 2 days prior to testing. The mice were divided into “medium first” and “odor first” dimension groups and tested every 2 days for set shifting using the method of Colacicco et al [36] and Garner et al [37]. The odors used were almond, maple, banana, vanilla, orange and lemon (all Club House brand). The media used were pieces of sponge, sand, Eppendorf tube lids, pro chip bedding, confetti and pieces of plastic straws. Mice were habituated for 10 minutes to a black acrylic chamber measuring $30 \times 45 \text{ cm}$ with a 15 cm divider in the center. Two days later, mice were again habituated for 10 minutes to the chamber, which contained two small plastic cups (diameter 5.5 cm, height 1.5 cm) baited with 10 small pieces of sugar and covered with bedding, one on each side of the divider. A piece of sugar was placed on top of each cup to encourage eating and digging for the sugar. Testing was done in six phases: simple discrimination (SD), compound discrimination (CD), compound discrimination

reversal (CDR), intra-dimensional shift (IDS), intra-dimensional shift reversal (IDSR) and extra-dimensional shift (EDS).

In the SD test, mice were exposed to only one dimension (medium or odor). In the medium condition mice were exposed to two media (e.g., sponge and confetti) and in the odor condition they were exposed to two odors (e.g., maple and orange), in which only one of the choices was rewarded with sugar. In the CD test, a second dimension was introduced (odor for medium first condition, media for the odor first condition), while the same dimension was still rewarded. For example, a mouse was still rewarded for digging in sponge and not confetti, but the bowls now had an odor (maple or orange). The combination of two dimensions was called an exemplar. For CD reversal, the odor or medium that was not rewarded the previous day was now rewarded. For the IDS phase, new media and odors were used to create new exemplars. The dimension rewarded in SD, CD and CDS was still rewarded (medium or odor). For IDS reversal, the odor or medium that was not rewarded the previous day was now rewarded. The last day was an EDS test, in which the relevant dimension was now reversed. Mice in the media first condition were now rewarded based on odor, and mice in the odor condition were now rewarded based on media. Mice were tested until they reached the criterion of six correct trials in a row, or 6 correct trials out of 8. In the first four trials each day, mice were allowed to dig in both bowls to find the sugar reward if their first choice was incorrect. After the fourth trial, incorrect choices marked the end of the trial and a new trial was started immediately. An increase in trials to criterion between IDS and EDS indicates that set shifting has occurred [37]. This task measured learning and attentional set-shifting abilities.

2.10. Social Preference & Social Recognition Tasks

Social preference and social recognition tasks were based on the procedure of Moy et al [19]. The apparatus consisted of a $69 \times 20 \times 20$ cm box made of 3-mm clear acrylic, divided by two walls into three compartments of equal size ($23 \times 20 \times 20$ cm). An opening (6×5.5 cm) located at floor level in each dividing wall allowed the mouse to move from the center into each end. The floor of the apparatus was covered with pro-chip bedding (1000ml) and acrylic doors were placed over the openings in the dividing walls. Mice were placed in separate cages for one hour and then habituated to the apparatus for 2 minutes, with the time spent in each chamber recorded. Immediately after habituation, mice were tested for sociability by being placed in the center chamber of the apparatus, with a novel stimulus mouse (a male CD-1 mouse from another colony room) placed in one chamber in a round wire cage (Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH) and a plastic pony (5×10 cm) placed in a Galaxy Cup in the opposite chamber. The dividing doors were lifted and the time the mouse spent exploring the social (novel stimulus mouse) and non-social (plastic pony) chambers was recorded over 5 minutes. The mouse was then removed from the apparatus and the apparatus was rotated 180 degrees to counteract any visual cues inside the apparatus. After a delay of approximately one minute, the mice were again placed into the center chamber of the apparatus with the stimulus mouse from the sociability test (familiar mouse) in one chamber and a novel stimulus mouse in the other chamber, both in the Galaxy cups. The divider doors were lifted and the time spent in the familiar and novel mouse chambers was recorded over 5 minutes. A percent time sociability preference and percent time social novelty preference were calculated using the times spent in the two end chambers. For a more precise measurement, a social preference ratio [time spent interacting with social cage/(time spent interacting with social cage + time spent interacting with non-social cage)] and a social novelty preference ratio [time spent interacting with novel mouse cage/(time spent interacting with novel mouse cage + time spent interacting with familiar mouse cage)] were calculated. This task measured social preference and social memory.

2.11. Cued & Contextual Fear Conditioning

Fear conditioning was done using the paradigm of Contarino et al [38] and Logue et al [39] using a MED Associates Inc. (St. Albans, VT) fear conditioning apparatus, which was housed in a room illuminated by a 60-Watt table lamp which decreased the glare from overhead lights on the Plexiglas walls of the chamber. The front, back and roof of the fear conditioning chamber (30.5 cm × 24.1 cm × 21 cm) were Plexiglas while the remaining two sides, one of which had a Med Associates speaker attached, were stainless steel. The fear conditioning chamber was placed within a sound-attenuating box (55.9 cm × 38.1 cm × 35.6 cm), with a clear polycarbonate door. A video camera was placed outside of the box so that it could record mouse activity within the chamber. Before each training and test session the grid rods and underlying floor of the fear conditioning chamber were cleaned with Sparkleen (Fisher Brand) dissolved in water.

For training, the mouse was placed into the fear conditioning chamber and the Plexiglas door fastened shut. Following a 120-second interval to record baseline freezing, the CS (auditory tone of 80-dB) was presented for 30-seconds. The US (2-second foot-shock of 0.7-mA) was administered 2-seconds prior to the termination of the CS. There was a 120-second interval, and then a second CS tone was presented for 30-seconds, co-terminating with the 2-second US (0.7-mA foot-shock). Thirty seconds after the administration of the second shock the mouse was removed from the test chamber and returned to its housing room. The total duration of the trial was 5.5-minutes.

Twenty-four hours after training mice were tested for contextual memory in the same chamber as fear conditioning training. Mice were re-introduced to the testing chamber for 5-minutes. The duration of freezing (immobile) behavior was recorded by a trained observer, blind to genotype, using a 10-second time-sampling method. No shock or tone was presented during contextual memory testing. The number of time samples in which freezing was recorded was converted to a percentage for analysis. One-hour following contextual fear memory testing, cued fear memory conditioning was tested in a second MED Associates testing chamber, which had black Plexiglas placed over the grid floor, the inside walls covered with black and white striped material and a novel odor (orange blossom, Club House Brand) placed on filter paper beneath the floor. Cued fear testing lasted 6-minutes for each mouse. The pre-CS three-minute segment during which baseline freezing to the new context was recorded, and the following three minute period where the CS (auditory tone) was presented continuously. The duration of freezing behavior was scored by a trained observer blind to genotype, using a 10-second time sampling method. The number of time samples in which freezing was recorded in the pre-CS and CS periods was converted to a percentage for analysis. This task measured cued and contextual fear memory.

3. Results

3.1. Elevated Plus-Maze

Due to tracking system errors, one male wildtype mouse and one female mutant mouse were not included in this analysis. There was no effect of genotype ($F(1,34) = 1.51$) or sex ($F(1,34) < 1.0$) on the proportion of time spent in the open arms or on the number of line crosses in the elevated plus-maze (genotype $F(1,34) = 1.59$; sex $F(1,34) < 1.0$; data in supplemental Table 1). There was no effect of genotype ($F(1,34) = 2.49$) or sex ($F(1,34) < 1.0$) on distance traveled, but there was a genotype by sex interaction ($F(1,34) = 5.75$, $p < 0.05$; Figure 1A). Sema5a mutant mice reared more than wildtype mice ($F(1,34) = 7.79$, $p < 0.01$), but there was no effect of sex ($F(1,34) < 1.0$; Figure 1B). Mutant mice had more head dips than wildtype mice ($F(1,34) = 5.03$, $p < 0.05$) and female mice had a greater number of head dips than male mice ($F(1,34) = 4.44$, $p < 0.05$; Figure 1C). Mutant mice had

significantly more stretch-attend postures than wildtype mice ($F(1,34) = 7.17, p < 0.05$), but there was no sex difference ($F(1,35) = 2.25$; Figure 1D). There was no effect of genotype on grooming ($F(1,34) = 2.68$; Table S1), but no effect of sex ($F(1,34) < 1.0$). There was no effect of genotype on freezing duration ($F(1,34) < 1.0$; Table S1), nor was there an effect of sex ($F(1,34) = 2.43$). There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on the number of defecations (Table S1), nor was there an effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) = 2.15$) on number of urinations (Table S1). There were no significant differences between socially and individually housed males on any of the behavioral measures.

3.2. Light/Dark Transition Box

There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on the proportion of time spent in the light zone of the light/dark transition box (Table S1). All mice spent more time in the dark than the light zone ($F(1,36) = 75.31, p < 0.0001$) (mean $\pm$ s.e.m. is 0.63 ± 0.02 for dark zone and 0.37 ± 0.02 for light zone). Mutant mice made more zone transitions than wildtype mice ($F(1,36) = 11.02, p < 0.01$), and females made more transitions than males ($F(1,36) = 6.24, p < 0.05$; Figure 1E). There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on the number of rears (Table S1). There was no effect of genotype on the number of stretch-attend postures ($F(1,36) < 1.0$), but females had more stretch-attend postures than males ($F(1,36) = 7.83, p < 0.01$; Figure S1). There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on grooming duration. There was no effect of genotype on time spent freezing ($F(1,36) = 1.55$), but females froze more than males ($F(1,36) = 3.55, p = .068$; mean $\pm$ s.e.m. is $0.77 \pm .33$ for males and 2.51 ± 0.86 for females). There was no effect of genotype ($F(1,36) = 2.88$) or sex ($F(1,36) = 2.06$) on number of defecations (Table S1). No urinations were observed. There was no effect of social separation on any of the behaviors of male mice in the light/dark transition box.

3.3. Open Field

There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on the number of line crosses and all mice decreased the number of line crosses on day 2 compared to day 1 ($F(1,36) = 55.95, p < 0.0001$) (mean $\pm$ s.e.m. is 140.6 ± 5.3 for day 1 and 94.2 ± 4.8 for day 2). There was no effect of genotype ($F(1,36) = 1.04$) or sex ($F(1,36) = 1.92$) on the activity change ratio (Table S1). There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) and no day effect ($F(1,36) < 1.0$) on time in the center, but there was a significant sex by day interaction ($F(1,36) = 5.01, p < 0.05$) as time in the center increased over days for males but decreased in females (Figure S2). There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on the frequency of rearing (Table S1). All mice decreased the amount of rearing on day two ($F(1,36) = 8.70, p < 0.01$). There was no effect of genotype ($F(1,36) = 1.49$), sex ($F(1,36) < 1.0$) or day ($F(1,36) < 1.0$) on stretch-attend frequency. The frequency of stretch-attend postures increased on day two for females but decreased for males ($F(1,36) = 17.18, p < 0.001$; Figure S3). There was no effect of genotype ($F(1,36) < 1.0$), sex ($F(1,36) = 2.76$) or day ($F(1,36) = 2.75$) on grooming duration. However, mutant mice increased grooming on day two while wildtype mice decreased grooming on day two ($F(1,36) = 5.94, p < 0.05$; Figure 1F). There was no effect of genotype on time spent freezing ($F(1,36) < 1.0$), but females froze more than males ($F(1,36) = 19.60, p < 0.0001$; Figure S4). There was a trend for more freezing on day 2 than day 1 ($F(1,36) = 3.46, p = 0.071$), and a sex by day interaction ($F(1,36) = 7.92, p < 0.01$; Figure S4). Mutant mice defecated more than wildtype mice in the open field ($F(1,36) = 5.41, p < 0.01$), and females defecated more than males ($F(1,36) = 5.41, p < 0.05$; Figure 1G), but there was no effect of day ($F(1,36) < 1.0$). There was no effect of genotype on number of urinations ($F(1,36) < 1.0$), nor day ($F(1,36) < 1.0$), but females did urinate more than males ($F(1,36) = 3.91, p = .056$; mean $\pm$ s.e.m. on day 1 was $0.15 \pm .1$ for males and $0.35 \pm .2$ and for

day 2, 0 ± 0 for males and $0.3 \pm .1$ for females). There was no effect of separation on the behavior of male mice in the open field.

3.4. Acoustic Startle & Prepulse Inhibition

There was no effect of genotype for acoustic startle amplitude (V_{max}) ($F(1,36) < 1.0$), but male mice had a greater startle response than female mice ($F(1,36) = 4.31$, $p < 0.05$; Figure S5). There was a significant effect of stimulus intensity ($F(7,252) = 78.00$, $p < 0.0001$) as the startle response was reduced as intensity increased from 74 to 90 dB, but there was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on percent prepulse inhibition. There was a significant effect of prepulse intensity ($F(4,144) = 130.87$, $p < 0.0001$; Figure 2A) as PPI increased as prepulse intensity increased. There was no effect of separation on the startle response or prepulse inhibition of male mice.

3.5. Rotarod

There was a significant effect of genotype on body weight (as measured on day 5 of the Rotarod) ($F(1,36) = 16.35$, $p < 0.001$), as mutant mice weighed more than wildtype mice. There was also a significant effect of sex ($F(1,36) = 121.00$, $p < 0.0001$) as males weighed significantly more than females (Figure 2B). There was no effect of genotype on latency to fall from the Rotarod ($F(1,36) < 1.0$), but there was a significant effect of sex ($F(1,36) = 16.65$, $p < 0.001$) as females were able to stay on the Rotarod longer than males. There was a significant sex by genotype interaction ($F(1,36) = 4.34$, $p < 0.05$) as male mutant mice took longer to fall than male wildtype mice, but female mutant mice performed worse than female wildtype mice. There was also a significant effect of day ($F(4,144) = 57.42$, $p < 0.0001$) as all mice showed an increased latency to fall over days (Figure 2C). Body weight significantly correlated with latency to fall from the Rotarod ($r = -.643$, $df = 38$, $p < 0.01$), indicating that the lighter mice stayed on the Rotarod longer than heavier mice. Analyzed separately, body weight was significantly correlated with latency to fall for females ($df = 18$, $r = -.521$, $p < 0.05$) but not for males ($df = 18$, $r = -.355$, ns). Group housed male mice weighed more than separated males ($F(1,16) = 5.62$, $p < 0.05$), but there were no effects of separation on the latency of male mice to fall from the Rotarod.

3.6. Visual Detection in the Visual Water Box

Sema5a mice performed better than wildtype mice in the visual water box ($F(1,36) = 5.63$, $p < 0.05$), and there was no sex difference ($F(1,36) < 1.0$). There was an effect of day ($F(7,252) = 17.74$, $p < 0.0001$) but no genotype by day interaction ($F(7,36) = 1.09$) as all mice performed better over days. All mice reached the criterion of 70% correct by day 8 (Figure 2D). Separated male mice performed better in the visual water box than group housed male mice ($F(1,16) = 6.25$, $p < 0.05$; Figure S6).

3.7. Morris Water Maze

There was no effect of genotype on latency to find the hidden platform in the Morris water maze ($F(1,36) < 1.0$) nor was there an effect of sex ($F(1,36) = 1.57$). There was a significant effect of day ($F(5,180) = 43.32$, $p < 0.0001$; Figure 2E) as all mice had shorter latencies over days. There was no effect of genotype on distance to the platform ($F(1,36) < 1.0$), but there was an effect of sex ($F(1,36) = 8.58$, $p < 0.01$) as females swam a longer distance to the platform than males (Figure S7). There was also a significant effect of day ($F(5,180) = 43.35$, $p < 0.0001$) as all mice swam shorter distances over days. There was an effect of genotype for swim speed ($F(1,36) = 5.50$, $p < 0.05$) as mutant mice swam faster than wildtype mice (Figure 2F). There was also an effect of sex ($F(1,36) = 5.62$, $p < 0.05$) as female mice swam faster than male mice. Although the sex by genotype interaction was not significant ($F(1,36) = 3.33$, $p = 0.076$), female mutant mice swam faster than all others.

There was no effect of genotype in the percentage of thigmotaxis (time spent within 10 cm of the edge of the pool), but there was an effect of sex ($F(1,36) = 4.51$, $p < 0.05$) as females spent more time in thigmotaxis than males and an effect of day ($F(5,180) = 120.11$, $p < 0.0001$) as the percent time in thigmotaxis decreased over days (Figure S8).

There was no effect of genotype on the percent time in each quadrant during the probe trial ($F(1,36) = 1.45$), nor was there an effect of sex ($F(1,36) = 1.45$), but there was a significant effect of quadrant ($F(3,108) = 77.70$, $p < 0.0001$) as all mice spent significantly more time in the “correct” quadrant (Table S1). There was no effect of genotype in the number of annulus crossings (crossing over where the platform would be) ($F(1,36) < 1.0$), nor was there an effect of sex ($F(1,36) = 1.16$; Table S1). There was no effect of genotype on the latency to the visible platform ($F(1,36) < 1.0$; Table S1), but there was a significant effect of sex ($F(1,36) = 5.71$, $p < 0.01$) as female mice reached the platform faster than male mice (Figure S9). There was a genotype by housing interaction for male mice in latency to the hidden platform ($F(1,16) = 5.53$, $p < 0.05$), as separated wildtype mice took longer than group housed wildtype mice, and mutant group housed mice took longer to reach the platform than separated mutant mice (Figure S10).

3.8. Set Shifting

There was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) = 2.26$) on the number of trials to criterion, but there was a significant effect of task ($F(5, 180) = 12.06$, $p < .0001$) (Figure 2G). A Fisher’s PLSD post-hoc test revealed that Simple Discrimination (SD) required more trials to reach criterion than all other tasks except Compound Discrimination Reversal (CDR). There was a significant effect of the dimension tested first on trials to criterion ($F(1,38) = 10.36$, $p < 0.01$) as mice trained with medium first required more trials to criterion than mice trained first with odor (Figure S11). There was a significant effect of task ($F(5,190) = 14.76$, $p < 0.0001$), and a significant task by first dimension interaction ($F(5,190) = 8.19$, $p < 0.0001$) as mice trained with odor first required more trials on SD, but then required fewer trials to reach criterion on all other days than mice trained first with medium. There was no effect of separation on the behavior of male mice in the set shifting task.

3.9. Social Preference & Social Recognition Tasks

In the social preference test, there was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on the percent time spent in the non-social and social chambers, but there was a significant effect of chamber ($F(1,36) = 18.65$, $p < 0.0001$) as all mice preferred the social chamber to the non-social chamber (Figure 3A). In the social recognition task, there was no effect of genotype ($F(1,36) < 1.0$) or sex ($F(1,36) < 1.0$) on percent time in the familiar and novel chambers. There was also no effect of chamber ($F(1,36) = 2.07$) as mice showed no significant preference for the novel mouse compared to the familiar mouse in the recognition task (Figure 3B). There was no effect of genotype on time spent in the center chamber for either the social preference task ($F(1,36) < 1.0$) or the social novelty preference task ($F(1,36) < 1.0$) and there was no effect of sex on time spent in the center for the social preference task ($F(1,36) = 1.01$) or the social novelty preference task ($F(1,36) = 1.59$). The large amount of time spent with the familiar mouse suggests that mice had not habituated to this mouse and so did not show an attraction to the novel mouse. There was no effect of separation on the behavior of male mice in the social preference or social novelty tasks. There was no effect of genotype on the social preference ratio ($F(1,36) < 1.0$), but females had a lower ratio than males ($F(1,36) = 6.45$, $p < .05$) (Figure 3C). There was no effect of genotype on the social novelty preference ratio ($F(1,36) < 1.0$), nor was there an effect of sex ($F(1,36) = 2.39$) (Figure 3D).

3.10. Cued & Contextual Fear Conditioning

There was no effect of genotype on the percent freezing in context ($F(1,36) < 1.0$), nor was there an effect of sex ($F(1,36) < 1.0$; Table S1). There was no effect of genotype on percent freezing in the cue test ($F(1,36) = 2.42$; Table S1), but there was a significant effect of sex ($F(1,36) = 7.48$, $p < 0.01$) as female mice froze more than male mice (Figure S12). There was a significant effect of CS presentation ($F(1,36) = 260.83$, $p < 0.0001$) as mice froze more during the CS (tone) than when no tone was present (mean $\pm$ s.e.m. is $13.1 \pm 1.4\%$ for pre-CS and $48.8 \pm 2.7\%$ for CS). There was also a significant CS by sex interaction ($F(1,36) = 9.48$, $p < 0.01$) as there was no sex difference in time freezing in the pre-tone period, but females froze more during the CS. There was no effect of separation on the behavior of male mice in cued and contextual fear conditioning task.

4. Discussion

While Weiss et al [9] found a reduced expression of *SEMA5A* in the brains of autistic patients, we did not find any evidence that *Sema5a* knockout mice would make a good model of autism (see Table 1). Crawley [16] has highlighted the behavioral abnormalities required in a mouse model of autism, including abnormal social interactions, deficits in communicative behavior, increased stereotyped repetitive behavior, anxiety, sensory hypo- or hypersensitivity and sleep disturbances. We did not find a lack of sociability in the *Sema5a* mutant mice, nor an “insistence on sameness” in the Morris water maze or set-shifting tasks, as all mice were able to learn the reversal rules. While *Sema5a* mutant mice showed increased risk assessment behavior in the elevated plus-maze, these anxiety-related behaviors were not found in the open field or light/dark box tests. *Sema5a* mice groomed more than wildtype mice in the open field, but wildtype mice groomed more than mutant mice in the elevated plus maze. We therefore did not find evidence of repeated stereotyped behavior in the *Sema5a* mutant mice, as they did not consistently differ from the wildtype in grooming or rearing behavior. Mutant mice showed similar results to wildtype in the prepulse inhibition task.

Sema5a mutant mice appeared more active and had more stretch-attend postures and head dips in the elevated plus-maze than C57BL/6 mice. *Sema5a* mice were also more active in the light/dark box, but not in the open field. Mutant mice defecated more than wildtype mice in the open field, indicating higher anxiety, although defecations were not increased in the elevated plus-maze or the light/dark box. Increased stretch-attend postures and head dips in the elevated plus-maze also suggest higher anxiety in the *Sema5a* mice compared to C57BL/6J mice, but no differences in anxiety-related behaviors were found in the light/dark box. In a triple test apparatus, combining the elevated plus-maze, light/dark box and open field, Fraser et al [40] found that the light zone of the light/dark box was the least aversive of the three tests to CD-1 mice, and mice preferred the elevated plus-maze to the open field. This suggests that the light/dark box may not be aversive enough to induce anxiety, which may explain why there was no difference in anxiety-related behaviors in this task compared to the elevated plus-maze and open field.

Both mutant and wildtype mice showed a preference for the social side in the social recognition task, but did not prefer social novelty. A lack of social novelty preference has also been found in C3H/HeJ, AKR/J, A/J and 129S1SvImJ mice, and of those, A/J and 129 mice did not show a social preference [20]. Pearson et al [41] found that C57BL/6J mice showed increased preference for social novelty only when they were placed in a novel context; i.e., by counterbalancing the location of the novel mouse in the social novelty preference task, which is generally not done, the preference for social novelty was lost. We rotated the apparatus between trials one and two, and so the location of the novel mouse was where the now familiar mouse had been located in trial one. If our mice were responding

more to context, this may have increased the amount of time spent with the familiar mouse in trial 2. We used outbred CD-1 male mice as stimuli, as they appear to be easier for mice to discriminate between than inbred strains [42]. Another social behavior related task, such as social transmission of food preference or free interactions between mice could confirm whether or not *Sema5a* mice have a social deficit.

Sema5a mutant mice did not show deficits in hearing (startle response and prepulse inhibition), vision (visual detection in the visual water box), olfaction (set shifting) or tactile discrimination (set shifting). This indicates that our results, particularly in the social preference/social novelty tasks, were not confounded by sensory deficits. Olfaction, rather than visual, tactile or auditory cues, seems to be the primary sense driving social preference in mice [43], and *Sema5a* mice were able to discriminate between odors as well as C57BL/6J mice in the set-shifting task. All mice were able to learn the Rotarod, indicating that these mice do not have a motor deficit. While there was a sex by genotype difference in the Rotarod, all mice improved over days. Mutant mice also swam faster than C57BL/6 mice. These results suggest that motor ability was not affected by semaphorin 5a knock out. There were no genotype effects in measures of learning and memory in the Morris water maze, set-shifting task or cued and contextual fear conditioning. Thus, a lack of semaphorin 5a did not adversely affect cognition, and these tasks were not confounded by sensory or motor deficits.

Unfortunately, the C57BL/6J mice that were used as controls were not littermates of the mutant *Sema5a* mice. Reduced maternal behavior has been shown to increase anxiety in adulthood in mice [44, 45]. If the mothers of the *Sema5a* mice were engaged in less maternal care than their C57BL/6J counterparts, it is possible that the increased anxiety observed in the *Sema5a* mice may be due to environmental factors rather than genetic factors, or some combination thereof. Future studies should look at maternal care and pup development in this strain.

Overall, most significant differences in this study appeared as sex effects, with females showing greater anxiety and males performing better in tests of learning and memory. There were sex by genotype differences in the Rotarod, suggesting a sex difference in balance and coordination differentially affected by *Sema5a*, but this difference is more likely due to differences in body weight. Surprisingly, social separation had no effect on social behavior in male mice, nor did it have an effect in the light/dark box or open field. Separated mice did show more risk assessment behavior in the elevated plus maze, and there were effects of separation on learning and memory in the Morris water maze and cued and contextual fear conditioning tasks. We are unsure as to why separated male mice performed better than group housed mice in the visual water box, as separation appeared to cause deficits in learning and memory, but all mice were able to reach criterion regardless of housing condition.

While our test battery was not exhaustive, there did not appear to be any conclusive behavioral deficits in *Sema5a* mutant mice. Plexin-B3 is the receptor for semaphorin 5A [46], and Plexin-B3 knockout mice have not been found to differ from their wildtype in measures of exploration, locomotion, motor coordination or anxiety [47]. A genetic association between Plexin-B3 and verbal performance in humans has been found [48], but Worzfeld et al [47] did not study social or communicative behavior in the Plexin-B3 knockout mice. It appears as though the semaphorin-plexin pathways are sufficiently redundant that a simple knockout of one protein is not sufficient to result in behavioral abnormalities, at least as measured in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Amanda O'Reilly, Joyce Yu, Ashley Whittaker and Nick Little for their assistance in behavioral testing. This experiment was funded by a NSERC grant to R.E.B and in part by NIH-NINDS R01NS059873 to M.J.H.

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Highlights

- Reduced Sema5A expression is associated with autism
- We examined a Semaphorin5A knockout mouse in a variety of behavioral tasks
- Sema5A mice appeared more anxious in the elevated plus-maze and open field
- Sema5A mice were more active
- There were no differences in cognition or social preference between Sema5A mice and wildtype
- Therefore we conclude that the Sema5A mice are not a suitable model of autism

Figure 1.

Anxiety and locomotion-related behavior. Mean $\pm$ s.e.m distance traveled (A); number of rears (B); head dips (C); and stretch-attend postures in the elevated plus maze (D). Mean $\pm$ s.e.m. number of transitions in the light/dark transition box (E); duration of grooming in the open field (F); and number of defecations in the open field (G). * p <0.05; ** p <0.01

Figure 2.

Sensory systems, motor systems and cognition. Mean percent ($\pm$ s.e.m.) prepulse inhibition to a 120 dB tone following prepulses of 74–120 dB (A); body weight on day 5 of the Rotarod (B); latency to fall from the Rotarod (C); percent correct in the visual water box (D); latency to the hidden platform (s) in the Morris water maze (E); swim speed in the Morris water maze (F); and trials to criterion in the set-shifting task (G). SD = simple discrimination, CD = compound discrimination, CDR = compound discrimination reversal, IDS = intradimensional shift, IDSR = intradimensional shift reversal, EDS = extradimensional shift. * $p < 0.05$; # $p < 0.001$; @ $p < 0.0001$

Figure 3.

Social behavior. Mean $\pm$ s.e.m percent time spent in the non-social and social chambers by Sema5a and C57BL/6J mice in the social preference task (A); percent time spent with the familiar and novel mouse in the social novelty preference task (B); social preference ratio using time spent interacting with wire cages (C); and social novelty ratio using time spent interacting with wire cages (D). *p<0.05; @p<0.0001

Table 1

Human symptoms of autism, with equivalent mouse behaviors and our findings with the Sema5a mutant mice (analogous mouse behavior based on Crawley [16] and Silverman et al [26]).

Human Symptom	Mouse Behavior	Findings with Sema 5a Mice
Core symptoms		
Inappropriate social interactions	Low social preference Low social novelty preference	Sema5a mice showed social preference Neither Sema5a nor C57BL/6J showed social novelty preference
Impairments in social communication	Low scent marking	No difference between genotypes in urinations in EPM, LDB or OF
Resistance to change	Large reversal effects in tests of learning and memory Inability to learn new rules	No difference in reversal learning from C57BL/6J No impairment in set-shifting task
Repetitive behavior, stereotypies	Repetitive/high levels of grooming Motor stereotypies (excessive rearing)	Sema5a increase grooming over days in OF while C57BL/6J decrease; Sema5a groom less in EPM Sema5a rear more in EPM
Associated symptoms		
Anxiety	High levels of time spent in “safe” areas of anxiety tests, high numbers of stretch-attend postures	Sema5a have more stretch-attend postures and head dips in EPM No differences between Sema5a and C57BL/6J in LDB or OF
Learning impairment	Poor performance in tests of learning and memory	No impairment in MWM, set-shifting, or cued and contextual fear conditioning
Clumsiness	Poor performance in motor and balance tasks	No impairment in Rotarod performance; Sema5a swim faster than C57BL/6J in MWM
Hyperreactivity to sensory stimuli	Impaired prepulse inhibition	No impairment

Abbreviations: EPM = elevated plus-maze

LDB = light/dark box

OF = open field

MWM = Morris water maze