Rat Behavioural Phenotyping Pipeline Methodologies

0	Animals, housing and husbandry

All animal procedures were undertaken in accordance with the University of Edinburgh animal welfare committee regulations and were performed under a United Kingdom Home Office project license. Long Evans heterozygous knockout rats were initially generated by the Medical College of Wisconsin gene Editing Rat Resource Center with support from the Simons Foundation Autism Research Initiative. Rats were then bred inhouse at the University of Edinburgh. Rats were genotyped by ear biopsy taken at P16±2 and weaned at P20±2 into same sex littermate groups. Rats were housed in polyethylene cages with wood chip bedding and enrichment (cardboard tube, aspen wooden gnawing block), typically with equal ratio of WT to TG littermates, in groups of 2 or 4 per cage. Rats were kept at 50% relative humidity in a 12h/12h light/ dark cycle with *ad libitum* access to water and from 7±2 wks of age maintained at 90±5% of their free feeding weight (typically 25g per day per male rat, 20g per day per female rat).
All behavioural experiments took place between 9:00am and 5:00pm during the light phase. Experimenters were blind to genotype and watched experiments on a computer screen in an anti-room, unless otherwise stated. Rats were transported to testing rooms at least 15 mins prior to testing.

	Pipeline Tests
1	General characterisation (protein, RNA levels)
2	Body weight
3	Neonatal reflexes
4	Object recognition memory
5	Object location memory
6	Marble interaction
7	Water maze spatial learning test
8	Active place avoidance
9	Auditory fear conditioning
10	Prey capture paradigm
11	Juvenile paired play paradigm
12	One trial adult social interaction

1 GENERAL CHARACTERISATION

Expression levels (mean ± SEM) of the target gene were measured using RNA sequencing, levels are expressed relative to the mean level in Wildtype (WT) samples, in prefrontal cortex (PFC) and hippocampal (HC) brain tissue samples. The characterisation at protein level was conducted with pups at P0 obtained from heterozygous*heterozygous cross. Pups are of mixed sexes, as they cannot be accurately sexed at P0. For RNA sequencing, samples were retrieved in male rats only, between 12-22 weeks old.

2 BODY WEIGHT

Pup weights were only taken when pups were completing the pup neonatal reflex paradigms and were typically measured at around postnatal day 3 (P3), P7, P10, P21. Adult body weights were taken from all rats undergoing behaviour, typically measured from 7 to 14 weeks of age. From approximately 7 wks of age rats were placed on a weight control diet to prevent obesity and encourage engagement in exploration and prey capture tasks. Male rats were fed 25g per day per rat and females 20g per day per rat. Rats were group housed and given food allowance daily on the cage floor.

3	NEONATAL REFLEXES

Righting Assay
Apparatus: A wooden plank (30 x 70 cm) was held horizontally on a aluminium frame (Kanya, UK) with a USB web cam (Logitech C922 Pro stream webcam, 1080p, 30fps) fixed in position 50 cm above and angled toward the center of the plank. Paper tissue was placed on the plank.

Procedure: photos of the pups' hair/skin colouring patterns, taken approximately every 4 days, were used to identifiy individuals across days. Pups were tested daily from P3 to P7, 5 days inclusive. The pups were all gently removed from the home cage and maintained in litter groups and placed in a clean holding cage on a heat pad for the duration of the test. The dam was kept in a separate holding room in her home cage. Trials were recorded using the webcam. Pups were tested in three 15 s trials per day (over 5 consecutive days), separated by a 20 s interval during which pups were held in a nitrile gloved hand to maintain body heat. On each trial, the pup was individually placed on its back on a piece of tissue and held in position for 2-3 s (Fig 3.1). The pup was then released, and the time it took to return to the prone position was recorded as righting latency (up to a maximum of 15 s).

'Righting ability' was defined as the animal being able to position itself with four paws on the ground within the 15 s trial. 'Righting success' was defined as the animal being able to right itself with all four paws fully on the surface for at least one attempt out of three, for two consecutive days. If the pup did not meet the criteria for success by P7, the daily testing would continue until they reached criterion. The second day in which the pup sucessfully righted for 1 out of 3 trials is reported as the day the pup reached criterion.

Figure 3.1. Righting reflex assay schematics. *Pup was placed on its back (left) and held in position for 2-3 s. Once released, the time the pup took to return to prone position (right) was recorded. The pup was given a total 15 s every trial, and if they had not returned to prone position during trial time, it was recorded as a failed trial.*

Negative Geotaxis Assay
Apparatus: A wooden plank (30 x 70 cm) was held at a 30° incline on a aluminium frame (Kanya, UK) with a USB web cam (Logitech C922 Pro stream webcam, 1080p, 30fps) fixed in position 50 cm above and angled toward the center of the slope. The wooden plank was covered with an aluminim perforated metal sheet (RS 202-7906, 2 mm holes, RS PRO, UK) with penlines to depict the angle the pup must reach in order to sucessfully complete the trial (Fig. 3.2A).

Procedure: approximately 30 min after the pup righting task ended (unless the pup had successfully completed the righting task e.g. from P7 onwards) the negative geotaxis assay was conducted. Between tasks, pups were kept in the holding cage on a heat pad in littermate groups. Pups were tested from P3 to P10, 8 days in total. Pups were individually placed with their heads pointing downward (180°) on the plank, which was inclined at 30°, with all four paws fully touching the surface (Fig. 3.2A,B). The pup was then released and left to attempt to turn up the slope. Pups that fell were immediately caught and returned to the start position (180°) this was repeated until the pup had nine falls, 45 s passed or the pup successfully turned. Successful turning was defined as the pup's head pointing up the slope within a 60° window (30° either side of 0°, which was completely pointing upwards, See Fig. 3.2B for pen lines that depict this window, and Fig 3.2C for successful pup trial). To reach criterion pups had to turn up in one attempt out of ten, for two consecutive days. If the pup did not reach criterion by P10, daily testing continued until they did. Time to successfully turn up and the number of attempts was recorded.

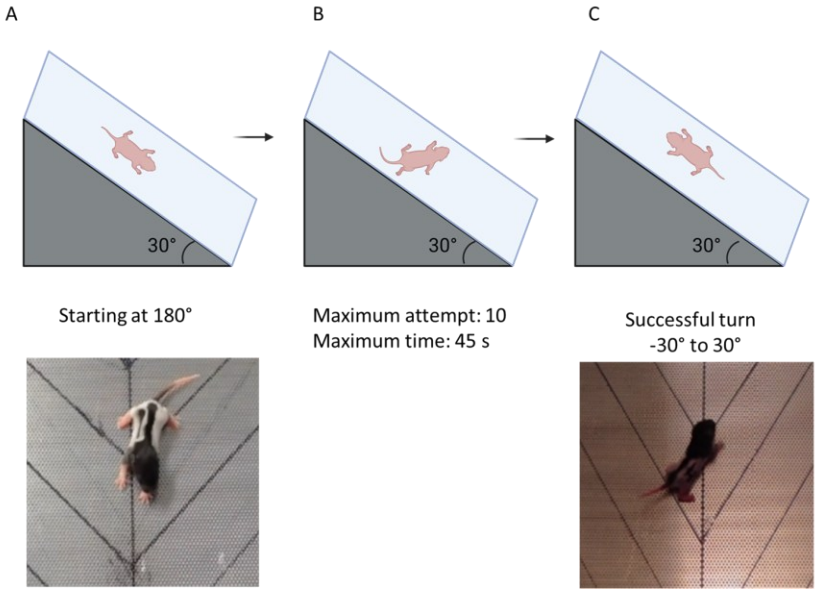


Figure 3.2. Negative geotaxis assay schematics (top row) and pictures (bottow row). A) *Pup at starting position (facing downward 180°).* ***B)*** *Pup attempts to right itself. If the pup fell, it was returned to the start position for a maximum of 10 attempts (i.e. 9 falls total), 45 s or until successfully turning.* ***C)*** *Successful turning was defined as the pup pointing its head upward on the incline to within a 60° window, 30° either side of 0°. If they had not returned to this position during trial time, it was recorded as fail. Piture below shows a successful trial by a pup at the end of the attempt.*

4	OBJECT RECOGNITION MEMORY

Edinburgh Methodology

Apparatus: for this task an arena (60x60 cm, 60 cm high, Kanya UK) with black laminated wood walls (60 cm) and a grey linoleum floor was used (Fig 4.1A). The light intensity was uniformly maintained at ~20 lx. No cues were placed within the arena and a black curtain pulled around the arena removed distal cues. Objects were secured in fixed locations within the arena using 3m Dualock tape. A CCTV camera fixed above the arena recorded all trials. An opaque black bucket (approx. 32 x 23 x 36 cm, with lid) with bedding from all home cages of test rats was used to hold rats between trials.

Procedure:
This task comprised of four stages: habituation, sampling, retention and test (Fig 4.1B). On the first day of habituation, rats were individually placed in the empty arena for 10 min before being returned to their home cages. This was repeated for four days. The arena was cleared of debris and wiped with 70% ethanol between rats. On the testing day (day 5), two identical objects (with no biological significance to rats e.g. ceramic tea lights, glass ornaments) were fixed in position (approximately 15 cm from the side and top edge of the arena). A rat was placed individually in the arena with the objects for a 15 min sample trial. The rat was then removed and placed in the holding bucket for 5 min. The objects were removed from the arena, the arena was cleaned with 70% ethanol and the objects replaced with a replica object and one novel object (counterbalanced for location and object between rats). The rat was returned to the arena for a 3 min test trial. To test long term memory, rats were returned to the arena 24 hr after the sample trial. To test short term memory, rats were returned to the arena 5 min after the sample trial.

Analysis: videos were scored manually using BORIS software (Friard, University of Torino) to quantify object exploration (defined as a rat directing its nose to an object within whisking distance, licking objects, placing head inside objects, climbing on with nose directed at object, but not climbing on and looking away from objects). A discrimination index (DI) for the exploration of the novel vs familiar object was calculated using the following formula: $DI = time_{novel} - time_{familiar} / time_{novel + familiar}$ (where time is the amount of exploration in seconds and novel is the new object and familiar is the object that was seen in the sample trial).
Parameters that were analysed in sample trials included total object exploration time in accumulating 1 min time bins (which could be partitioned into time exploring the object on left/right or object identity to look for for object/side bias). In the test trials we analysed total object exploration time and time exploring the novel/familiar object in 10 s accumulating time bins. Discrimination index was also plotted for 10 s accumulating time bins, and after 10, 15, 20 25 s of accumulated object exploration.

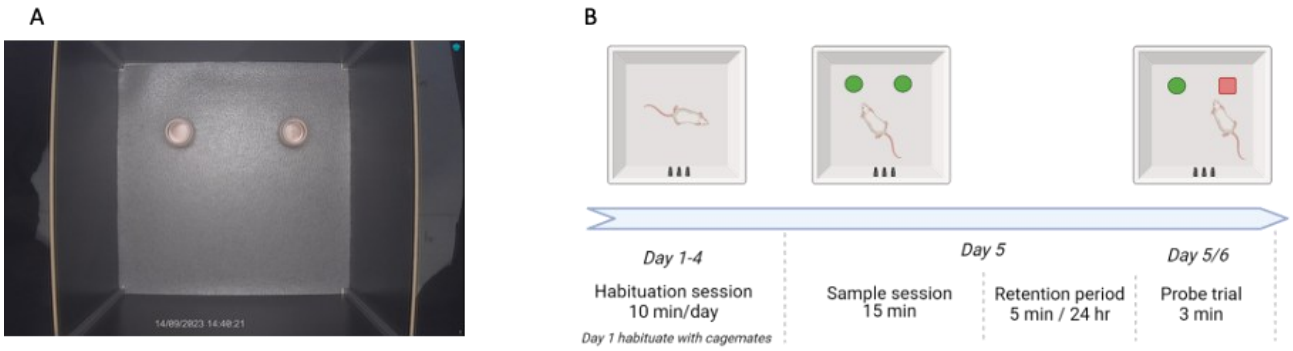


Figure 5.1 Object recognition task (Edinburgh) arena and schematics. A) *the arena, showing two identical objects and no arena cues* ***B)*** *Animals received habituation on day 1-4. On day 5, rats explored 2 identical objects in a 15-min sample session. Object recognition performance was then assessed 5 min later or 24 hours later in the probe trial, where one of the two objects was replaced with a novel one and the other object was replaced with a replica object.*

Bangalore Methodology

Apparatus: The same arena design as used in Edinburgh was used. The box was made of laminated wood, measuring 60cm in length and width, and 60cm in height. The arena featured white walls and was filled with a layer of corncob bedding, 3cm deep. At the centre of one wall, a cue card displaying 1-inch thick vertical black and white stripes was strategically positioned; this served as the only visible cue within the arena. The light intensity in the arena was approximately 20 lx. A top-mounted infrared camera captured detailed video recordings of the animals as they navigated the space.

Procedure: To help minimise stress and prepare the animals for the experiment, they were relocated to a designated cupboard that provided adequate light and ventilation for 30 minutes before the start of the task. The experimental protocol comprised four phases: habituation, sampling, retention and probe (Fig 4.2). For three consecutive days (Days 1-3), each animal was introduced to the empty arena for 10 minutes daily (habituation phase). This allowed the animals to familiarise themselves with their surroundings without the presence of any objects. Compared to the Edinburgh protocol, there was no group habituation session before the three individual habituation sessions. Next, for days 4, rats were placed in the arena with two identical ceramic objects to explore for a total of 10 minutes (this was different from the Edinburgh protocol). The objects were arranged symmetrically against one of the walls. After the sampling phase, rats were left undisturbed for a retention period depending on the versions of the task. For assessing short-term memory, rats were kept in the holding cage for 5 minutes and reintroduced to the arena for the probe trial. For assessing long-term memory, rats were left undisturbed in their home cage for 24 hours, then received the probe trial on the next day. During the probe trial, one of the identical objects from the sampling phase was replaced by a different object, and the rats were allowed to explore for 3 minutes during this trial.

Analysis: The videos recorded during the experiments were analysed using Noldus Ethovision (Observer XT) software, which provided quantifiable measures of exploratory activity, including total distance travelled (cm), average velocity (cm/s) and time spent immobilised (s). The object interactions during the probe trials were evaluated using z-score analysis software developed by Professor Oliver Hardt. Specific attention was given to active exploratory behaviours, such as touching the objects with their noses or front paws, licking, sitting on the objects while actively exploring, and poking their heads or noses into the holes. Conversely, certain behaviours, such as supported rearing (where the animal took support from the object while looking away) and unsupported rearing (where the animal sat on top of the object while looking away), were systematically excluded from the analysis to maintain the integrity of the exploratory activity measurements.

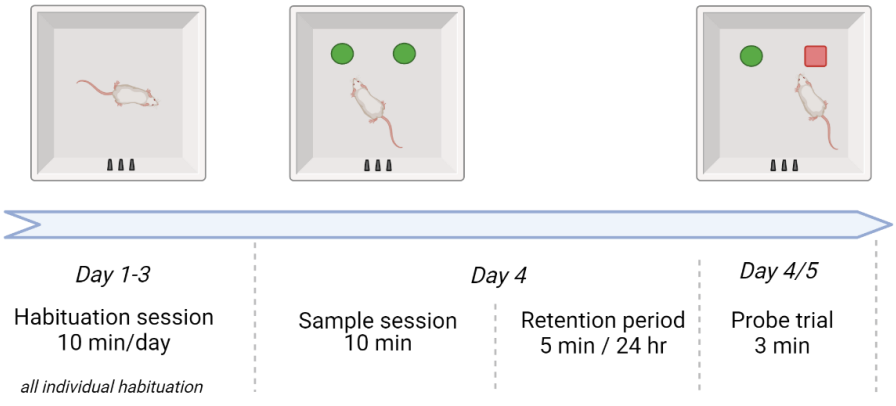


Figure 4.2. Object recognition task (Bangalore) schematics. *Animals received habituation on day 1-3, all of which were individual habituations. On day 4, rats explored 2 identical objects in a 10-min sample session. Object recognition performance was then assessed 5 min later or 24 hours later in the probe trial, where one of the two objects was replaced with a novel one and the other object was replaced with a replica object.*

5	OBJECT LOCATION MEMORY

Edinburgh Methodology

Apparatus: for this task two arenas (60 x 60 cm, 60cm high Kanya UK), located in different rooms were used. The small room arena had black laminated wood walls and fresh woodchip bedding on the floor, an A4 black and white cue on the north wall of the arena, and two cues attached at both NE and NW corners (see Fig 5.1A). The big room arena had white laminated wood walls and white nesting bedding on the floor, an A4 black and white cue on the north wall of the arena, and two cues attached at both NE and NW corner (see Fig 5.1B). Rats were randomly assigned to be tested for long term memory in one arena and short-term memory in the other, counterbalanced across genotype (and if applicable, sex). The light intensity in the arenas was uniformly maintained at ~20 lx, measured at the center of the floor. Objects were secured in fixed locations within the arena using 3m Dualock tape approximately 10 cm from the sides of the arena. A CCTV camera fixed above the arenas recorded all trials. An opaque black bucket (approx. 32 x 23 x 36 cm, with lid) with bedding from all home cages of test rats was used to hold rats between trials.

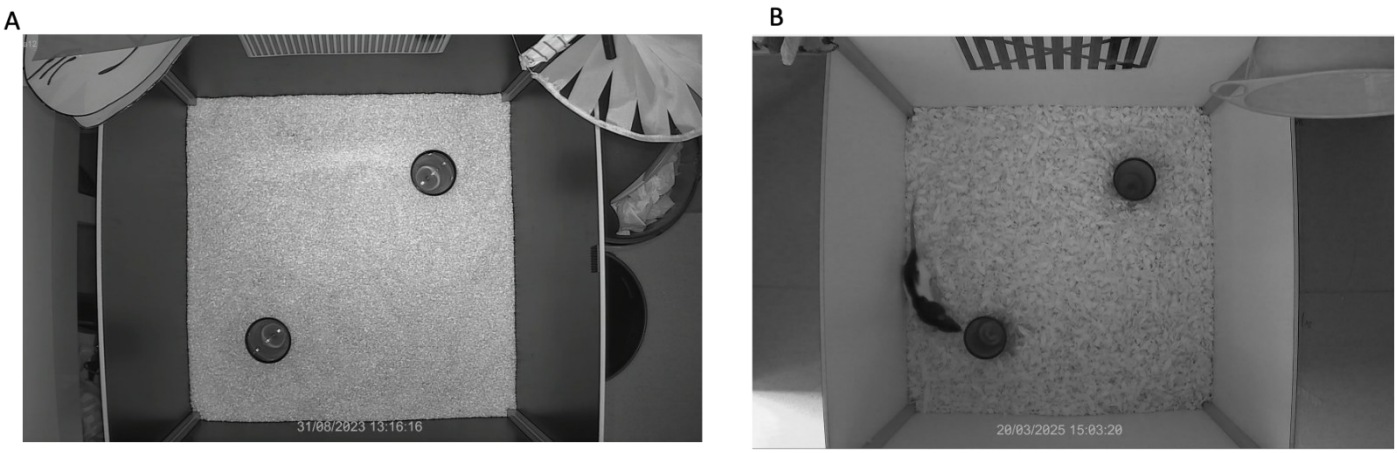


Figure 5.1 Screenshots of video recordings during the Object Location Task in the Edinburgh pipeline. A) *the small room arena: black laminated wood walls and fresh woodchip bedding on the floor, an A4 black and white cue on the north wall of the arena, and two cues attached at both NE and NW corners and* ***B)*** *the big room arena: white laminated wood walls and white nesting bedding on the floor, an A4 black and white cue on the north wall of the arena, and two cues attached at both NE and NW corner.*

Procedures: The habituation phase consisted of 3 days of one daily trial. On day 1 rats were placed in their assigned arena together with cagemates (typically 4) for 10 min. For the next two days, rats were placed in the arena individually for 10 min.

On day 4, two identical copies of an everyday object with no biological significance for rats (e.g., ceramic tea lights, glass ornaments, etc.) were fixed in diagonally opposing corners of the arena. One rat was placed in the arena, facing a corner not containing an object (start position was predetermined and counterbalanced across genotype, and was different for sample and test trial), and remained in the arena for a 15 min sampling trial. The rat was then removed and placed in the holding bucket for 5 min. The objects were removed from the arena and wiped with 70% ethanol and thoroughly dried. The arena floor was cleared of debris, the bedding then mixed and levelled. The objects were returned to the open field arena with one in the same location as during the sampling trial and one placed at a different location than before, i.e., at the middle of a wall (see Fig 5.2; the object that moved was counterbalanced across genotype). The rat was returned to the arena in its predetermined start position for a 3 min test trial. To test short-term object location memory the test trial took place 5 min after sampling, and 1 h after sampling to test long term memory.

Analyses: videos of sampling and test trials were scored using BORIS software (Friard, University of Torino) to quantify object exploration (defined as a rat directing its nose to an object within whisking distance, licking objects, placing head inside objects, climbing on with nose directed at object, but not climbing on with nose directed away from objects). The Boris software generated an xcel file with time stamp data of object exploration and Matlab code (version R2022b) was then used to convert the excel file into a text file for use with zChop (Oliver Hardt, McGill University). ZChop was used to calculate a discrimination index (DI) for the exploration of the novel vs familiar location of the objects for different time intervals of the test trial. DI was calculated using the following formula: $DI = time_{novel} - time_{familiar} / time_{novel + familiar}$,where time is the amount of exploration in seconds of the object at the novel or familiar location, respectively. Total object exploration time was also calculated for the sample and test trials.

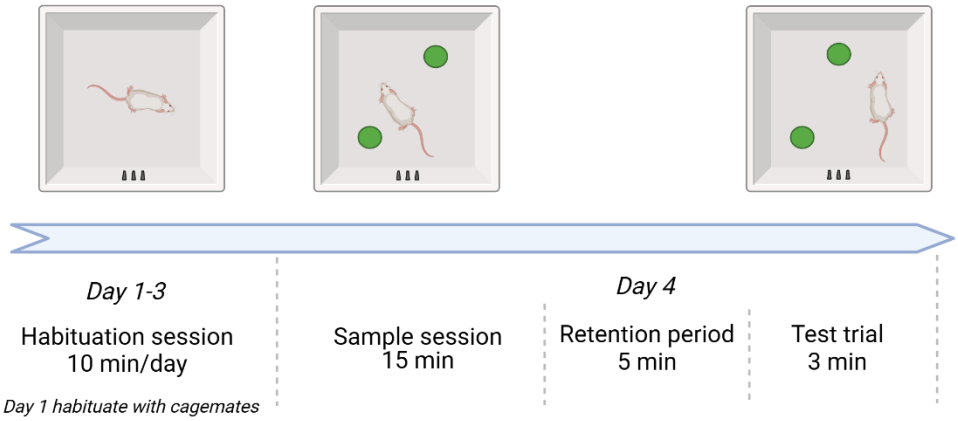


Figure 5.2. Object location task, short term memory (Edinburgh) schematics. *Animals received habituation with cage mates for 10 min on day 1, and individual habituation on day 2 and 3. On day 4, rats explored 2 identical objects placed diagonally for 15 min. They were then placed in a holding bucket for 5 min. After which they received the testing trial for 3 min in the same arena with one of the objects displaced to a novel location.*

Bangalore Methodology

The Bangalore pipeline used the same apparatus as the Edinburgh pipeline. However, this pipeline aimed to look at memory retention over a longer period of time than the Edinburgh pipeline (3 days instead of 1 hr).

Apparatus: as used in Edinburgh.

Procedures: Thirty minutes before the experimental trials began, animals were placed in individual cages placed in a temperature and light controlled holding chamber in the ante room of the experimental room.

The experimental protocol comprised four phases: habituation, sampling, retention time, and probe (see Fig. 5.3). For four consecutive days (Days 1-4), each animal was introduced alone to the empty arena for 10 minutes daily (habituation phase). Next, the sampling phase occurred: days 5 –7, rats were put in the arena with two identical copies of an object, placed in diagonally opposing corners. Each sampling trial lasted 10 min or was aborted when rats reached 60 s of object exploration time, whichever came first. After the last day of sampling, rats remained in their home cages for a three-day retention period (days 8–10). On Day 11, rats were returned to the arena for the 3-min test trial, where one of the objects from the sampling phase was relocated to the middle of the wall, with the other object remaining in its original location.

Analyses: The videos recorded were analysed using Noldus Ethovision (Observer XT) software, to track total distance travelled (cm), average velocity (cm/s) and time spent immobilised (s). During the sampling phase, the cumulative 60 seconds of object exploration was documented manually by the experimenter in real time. Object exploration was scored with zScore (Oliver Hardt, McGill University). A rat was considered exploring an object when its snout was within 2 cm of the object and oriented at an angle of at least 45 degrees toward it. Climbing and resting on the object was not considered exploratory behavior.

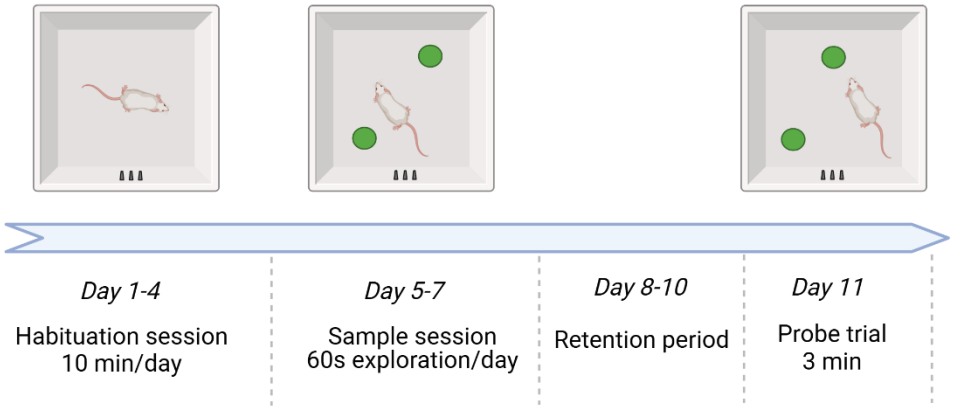


Figure 5.3. Object location task (Bangalore) schematics. *Animals received habituation for 10 min daily on day 1 to day 3. On day 5 to day 7, rats explored two identical objects for 60s per day. The objects were placed diagonally to each other. On day 8-10, rats received no training or testing. On day 11, one of the objects in the corner was moved to the middle of the edge wall. The rat was then put back to the arena for a 3-min probe trial.*

6 MARBLE INTERACTION

Apparatus: The arena consisted of a plastic box identical to the rats' home cage base (54 x 36 x 20 cm) with 5 cm of wood chip bedding on the floor. The cage bases had white plastic panels slotted into a frame around the bases on each side to a total height of 71 cm (Fig. 6A). Two arenas were placed side by side with a wooden panel between the two arenas to prevent visual contact between rats, so that two animals could be tested simultaneously. The light intensity was ~20 lx in the arenas. Twenty black shiny glass marbles (25 mm, Opal marble, House of Marbles, UK) were used. A CCTV camera mounted on the ceiling above each arena recorded the trials.

Procedures: On day 1 and 2 rats were placed individually in the arena for 20 min with wood chip bedding and no marbles. Between rats the arena was cleared of debris and the wood chip bedding mixed and flattened out. On day 3, the rat was placed in the arena with 20 marbles positioned in a 4x5 grid on top of the wood chip bedding for 20 min (Fig. 6B). Between rats the marbles were washed with dish soap and water and dried.

Analysis: Parameters analyzed included the time spent manipulating marbles using front paws, manually scored from the videos using BORIS software (Friard, University of Torino). Distance moved was tracked using ANY-Maze (Stoelting Europe, Dublin).

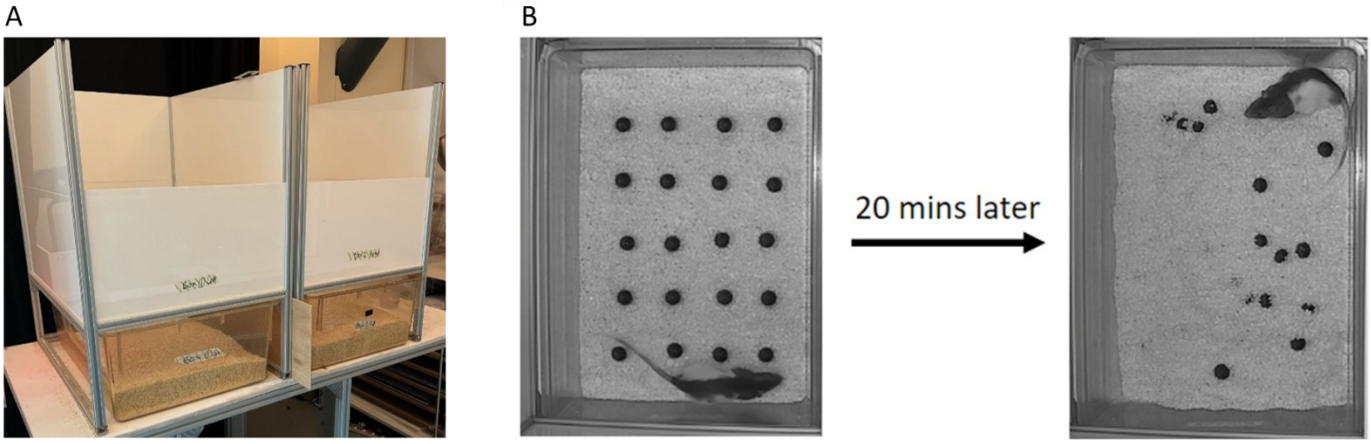


Figure 6. Marble interaction task set-up (A) and picture of a day 3 testing session (B). A) *The set-up consisted of two testing cambers for simultaneous testing of two rats. All four sides of the arena had white panels. The topmost panel is removed for this photo but reached total height of 71 cm from base.* ***B)*** *Rats were placed the arena with 20 marbles arranged in a 4x5 grid. Images showing an example testing trial before and after the 20 min of testing. Interaction with the marbles with front paws was measured.*

7	WATER MAZE

Apparatus: The water maze consisted of a circular pool (2 m diameter) with spatial extra-maze parallax cues provided by large objects hanging from the ceiling and walls (Fig. 7.1). The distance from the pool rim to the nearest wall was 77 cm and to the farthest wall was 125 cm. A white curtain could be pulled entirely around the pool to hide the extra maze cues. The pool water was made opaque using approximately 200 ml of liquid latex and maintained at a temperature of 28-29°C. An Atlantis platform, (10 cm in diameter; Spooner et al., 1994) which could be programmed to rise from the bottom of the pool at a predetermined time to become available to the swimming rat, was used in daily probe trial as the first trial of each day to test long term memory retention for the platform's location on the previous day. The platform was unavailable for 60 s, then rose up to 1 cm below the surface of the water so that rats could climb on it (thus avoiding extinction of the platform's location; Spooner et al., 1994). The platform remained 1-2 cm below the surface during all other trials of the day.
A camera mounted 155 cm above the water surface over the centre of the pool recorded all trials. Video tracking software (WaterMaze, formerly ActiMetrix, now a subsidiary of Lafayette Instrument Company, USA) was used to run and analyse the trials. Home cages were placed on heat pads throughout the experiment and rats were towel dried immediately after each swim. The experimenter observed the trials from behind a partitioning wall.

Figure 7.1. Picture of the water maze showing the extra-maze distal cues hanging on or in front of the walls.

Procedures: 'Visible platform trials' were conducted first to assess vision, swimming ability and demonstrate to the rats that the platform is the only means of escape (Fig. 7.2A). The platform was raised 1-2 cm above water level and had an object placed on it as a proximal cue. A curtain was drawn around the pool to remove the extra maze cues. The rats were given 4 trials per day with a maximum duration of 2 min, with an inter-trial time of approximately 15 min. The platform moved between trials (either NE, NW, SE, SW quadrant, approximately 70 cm from the pool rim) and the rat was placed into the pool at a different release point for each trial (N, E, S, W). After the rat found the platform, it was left for 20 s before being carefully lifted, towel dried and returned to its home cage. Rats that did not find the platform were guided to it and left on it for 20 s.

Next, long term spatial memory was assessed by keeping the platform in the same location over days (either NE or SW quadrant, 70 cm from the edge), approximately 1-2 cm below water level with extra maze cues available to the rats (Fig. 7.2B). This protocol was conducted for 4 days with 4 trials each day (see Fig 7.2D). Each trial was 2 min max in duration. Each of the 4 daily trials started from a different location (N, E, S, W). After the rat found the platform, it was left for 20 s before being carefully lifted, towel dried and returned to its home cage. Inter-trial time was approximately 15 min. However, the platform was not present for the first 60 s of the trial, during which dwell time in the target area could be quantified to assess long term memory for the platform location. The platform was raised automatically after 60 s and the rat had a further 60 s to locate and climb onto it (thus preventing extinction of the platform's location).

Finally, to assess reversal learning – a form of cognitive flexibility –rats were tested in trials in which the platform was located in the opposite quadrant than the previous days (e.g., if in NE, it is now in SW; Fig. 7.2C). The same protocol as for long term memory testing was used over 4 days of testing. During the first swim of each day, in which the platform was not present for the first 60 s, the dwell time in the new and old target area was quantified over days to assess extinction of the old and learning of the new location.

Analyses: Parameters that were analysed included path length, trial duration, swim speed, dwell time in quadrants, % thigmotaxis (time in outer 12 cm perimeter), all automatically generated by the Actimetrix tracking software.

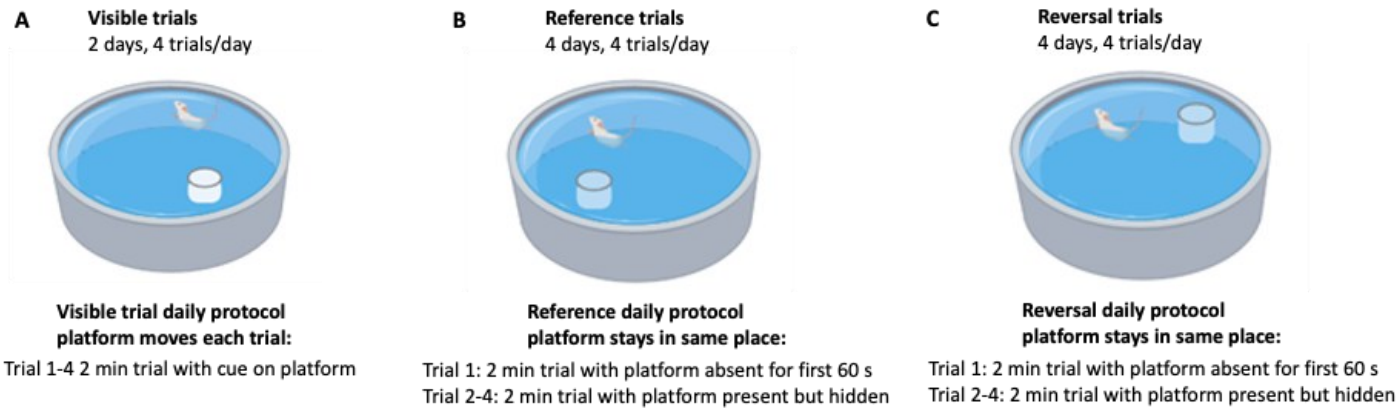


Figure 7.2. Morris Water Maze Schematics. A) *in visible platform trials the platform was 1-2 cm above the water surface, moved between trials, and spatial cues were not available;* ***B)*** *in long term spatial memory testing the platform remained in the same location over days and was 1-2 cm below water level. Spatial cues were available. In the first swim of each day the platform only became available after 1 min.* ***C)*** *in reversal learning trials the location of platform was moved to the opposite quadrant compared to prior trials. In the first swim of each day the platform only became available after 1 min.*

8	ACTIVE PLACE AVOIDANCE

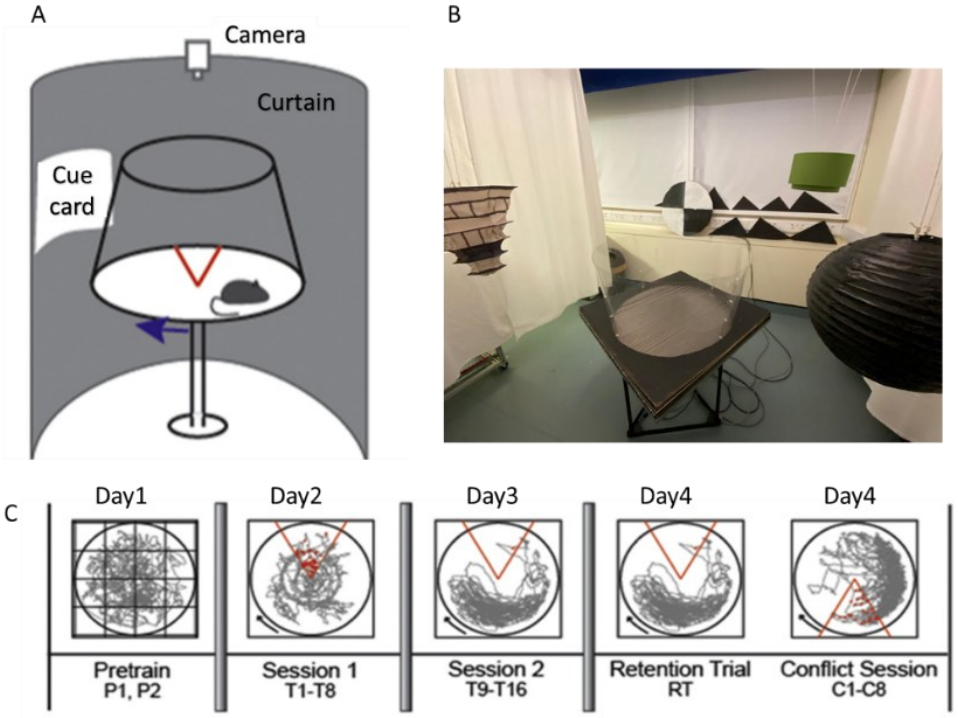
Apparatus: The apparatus consisted of a rotating rectangular grid floor (100 x 100 cm) placed 90 cm above the ground (Biosignal group, Brooklyn, USA). Light levels were maintained at ~x lx. A transparent plastic wall placed onto the rotating grid floor created a circular arena (80 cm diameter, 40 cm high; Fig 8A). Electric shocks could be delivered through the grid floor. Foot shock (strength 0.2 mA, for 500 ms, at a frequency of 1.5 s) was delivered as long as the rat was within a 60° shock zone located in the North or South region (counterbalanced between rats). A white curtain approximately 3 m from center of the arena was pulled around the N, E and W side leaving the S side open to the wall which had black and white cues positioned on it (See Fig 8B). In addition, 3 different shaped hanging lamp shades/lanterns hanging approximately 1-2 m from the center of the arena provided spatial cues around the maze (see Fig 8.1B). An overhead camera recorded animal behaviour which was then analysed with the tracker software (Biosignal group, Brooklyn, USA). An opaque black bucket (approx. 32 x 23 x 36 cm, with lid) with bedding from all home cages of test rats was used to hold rats between trials. The experimenter watched the trials on a computer screen from behind the curtain.

Procedures: In pretraining trials rats received two trials (P1 - P2) on one day, each trial 10 min long with 10 min between trials, during which the rat was placed in a holding bucket (Fig 8C). During the trial the arena was not rotating, and no shocks were administered. The rat started the trial from the N or S quadrant.

Next, during training, rats received 8 trials per day for 2 days (T1 - T16), each trial 10 min long with 10 min between trials, during which the rat was placed in a holding bucket. The arena rotated at 1.5 RPM and a shock was administered when the rat entered the shock zone. At the start of each trial the rat was placed in the quadrant opposite to the shock zone.

Long-term retention of for the shock zone location was assessed 24 h after the last training trial in a 10 min trial (R1) in which no shocks were administered and the arena rotated at 1.5 RPM. The retention trial was followed by 8 conflict trials (C1 - C8), in which rats received shocks for entering the quadrant opposite the previously learned zone (e.g. if zone was in the N, it was then in the S). The arena rotated at 1.5 RPM and the inter trial interval was 10 min.

Analyses: Post acquisition, the room and arena track .dat files were analysed in using the Track Explorer software to generate the following parameters: total path length, number of shocks received, time to first shock, time to second shock, number of entries to shock zone, max time avoided shock zone, for all relevant trials.



.

Figure 8. Active Place Avoidance schematics. A) diagram showing the experimental setup. *A rotating arena was set up, with a shock zone of 60° in the N or S. Various distal cues were present in the room. The overhead camera tracked the animal's location and shocks were automatically delivered when the animal entered the "active" zone (red wedge in A and C), which was spatially defined by its location relative to the room cues.* ***B) Photo of the experimental room*** *showing the location of distal cues in relation to the arena.* ***C) Representative traces of animal movement during the trials of the protocol.*** *The "active" zone (red wedge) remained at the same location, relative to the room cues, during the training trial but was moved to the opposite side of the platform during the conflict sessions (i.e., for reversal learning). Note that no shock was delivered during the pretraining or retention trials*

9	AUDITORY FEAR CONDITIONING

Apparatus: Two different Coulbourn Fear conditioning chambers (Coulbourn, USA) were placed within sound-attenuating chambers (Coulbourn, USA) to provide two distinct contexts (A and B, Fig. 9). Context A had a smooth plastic flooring, a curved black and white striped plastic mat across two walls and one wall had black and white tiles. Mint toothpaste (Colgate stripe) was placed in a dish outside the box within the sound attenuating chamber to provide an odour cue. Context B had a grid shock delivery floor, all 3 walls had shiny steel tiles, and no mint odour was present. FreezeFrame 5 software (Actimetrics, Lafayette USA) was used to administer the shock/cues, record trials, and analyse freezing levels (defined as cessation of all behaviour except that associated with breathing). The contexts were replicated, and two rats were run simultaneously.

Procedures: on Day 1 and 2 rats were placed in context A for 20 min for habituation. Context A was cleaned with 70% ethanol between rats. On Day 3 rats underwent auditory fear conditioning. Rats were placed in context B, and after 2 min received the first of three tone-shock parings spread 1 min apart (30 s duration, 5 kHz, 75-80 dB, co-terminating with a 0.7 mA scrambled foot shock of 1 s duration. Following the last tone-shock pairing, rats remained in the box for a 2 min before being removed and placed in a holding cage until all cage mates were tested. Context B was cleaned with Sanisafe 4C Antibacterial Anti-Viral Wipes between rats. On Day 4 and 5 rats were tested for recall of the conditioned response in a standard extinction protocol. Rats were placed in context A and after 2 min received 13 presentations of the tone (30 s duration, inter tone duration 30 s, 5 kHz, 75-80 dB); foot shocks were absent. Following the 13th tone presentation, rats remained for a further 2 min before being removed and placed in a holding cage until all cage mates were tested and then returned to home cage. Context A was cleaned with 70% ethanol between rats.

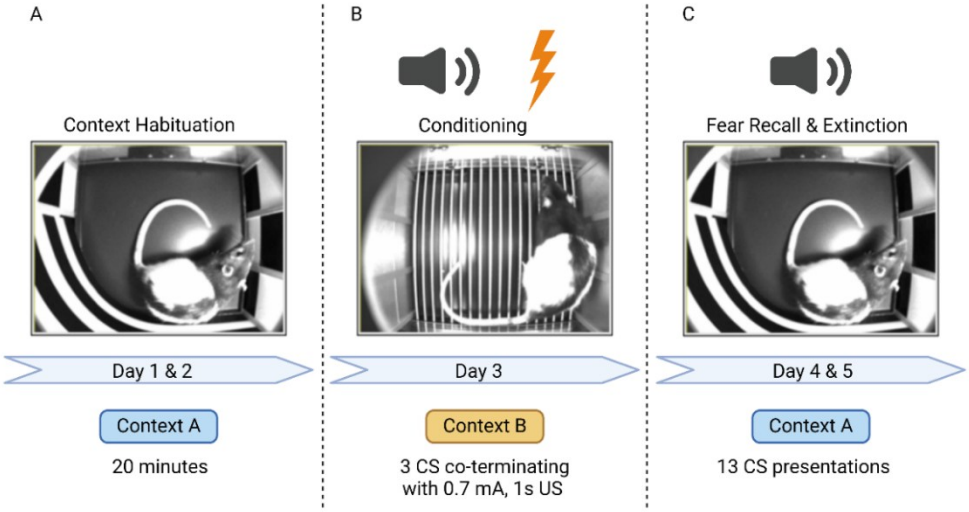


Figure 9. Auditory Fear Conditioning Schematics A) Day 1-2 habituation*: animals placed in context A, received no tone or foot shock;* ***B) Day 3 Conditioning****: in context B, animals received 3 tones with co-terminating foot shock of 0.7mA for 1 s* ***C) Day 4-5 Fear Recall and Extinction****: animals received only tone but no foot shock in context A.*

Analyses: All trials were recorded. FreezeFrame 5 software was used to perform analysis and SMP was used to score freezing behaviour (cessation of all movement apart from that associated with breathing). Scorers manually set thresholds for freezing detection for each experiment. The threshold was set to 0 for all habituation trials. For conditioning and recall trials, a variable threshold was determined for each individual rat based on their activity/movement. The software was used to extract % time freezing in time bins for baseline, tone presentation, and the period between tone presentations (ITI, inter-tone-interval).

10	PREY CAPTURE

Apparatus: The testing apparatus consisted of a plastic square arena (100 x 100 cm; PVC) with 50 cm high white walls and a wood (MDF) lined floor painted white (to increase grip for crickets) with lighting maintained at approx. 75 lux. A CCTV camera was positioned directly overhead to record all trials. Rats were held in an opaque starting cylinder (diameter approx. 20 cm, height 25 cm; removable lid) inside the arena before trials; between trials, rats were placed into a black opaque holding bucket (approx. 32 x 23 x 36 cm) with a lid and bedding from each of the rats' home cage outside the arena in the adjoining ante room on top of a workbench.

Live silent brown crickets (25-30 mm; bought fresh for each experiment from LiveFoodsDirect UK) were maintained in 2 large plastic boxes (145L box, 80x60x40 cm, with holes drilled into the lid) for durations of 2 days to a maximum of 2 weeks before being used in an experiment. The cricket boxes held up to 250 crickets maximum. Crickets were housed with access to hydration gel (ProRep bug gel, LiveFoods Direct, UK) and fresh food (e.g. carrot, potatoes, bran flakes, cat biscuits) and only healthy crickets (no signs of disease, body/legs intact) were used in the experiments. The number of crickets required for the day's experiment (e.g. approximately 50) were placed in a smaller container (Exo Terra Faunarium, Swell Reptiles Ltd UK, 30 x 20 x 20 cm) and placed on a heat mat for at least 10 min prior to use, to encourage fleeing. The cricket holding chamber would stay on the heat mat for up to 4 hours for the entire duration of an experiment. Insect holding tongs (Bug tongs, Forest School Shop UK) were used to manipulate crickets.

Procedure: Rats were habituated over five consecutive days to the testing arena and behavioural procedures. On Day 1, cage mates were placed in the arena together for 30 min. On Days 2-5, rats were placed individually in the arena for 10 min. On Day 3, rats were placed individually in the arena in a starting cylinder (Fig 10A) for 15 s before being released into the arena (the holding cylinder was lifted manually to release the rat) with a dead cricket (*n.b.* dead crickets may be able to twitch a long time after death, but they would not be able to flee) placed in the opposite corner to the rat. The rat was allowed 10 min to explore and potentially interact with or consume the dead cricket. Days 4 and 5 were the same as day 3 with the addition of placing the rat into a holding bucket in the adjoining ante room for 2 min after the trial. Between all trials the arena was cleaned and wiped with water. The experimenter watched the trials on a screen from an anti-room.

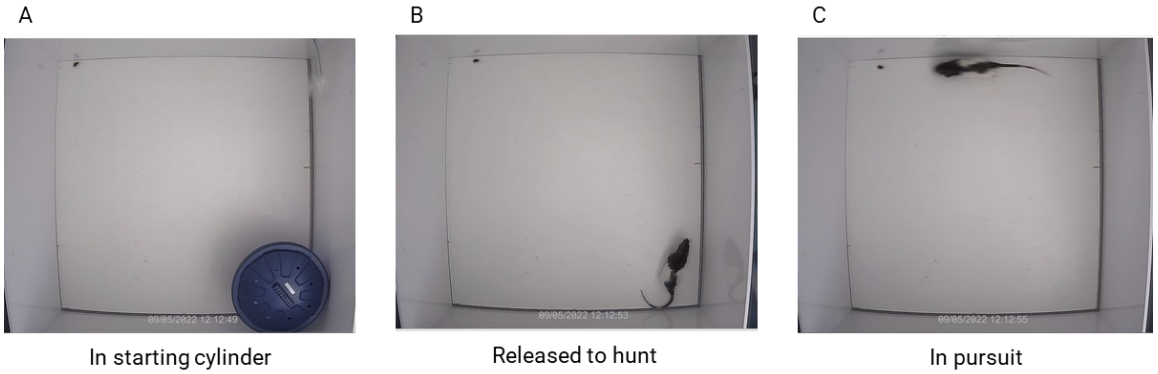


Figure 10. Prey Capture Schematics A) Image of a starting cylinder in the arena. *From day 3 onwards, before each trial, the rat was kept in a starting cylinder for 15 seconds as the dead/live cricket was placed in the arena;* ***B) Image of a rat just released from the start cylinder.*** *The rat was then given 2 min to hunt for the cricket;* ***C) Example picture showing a rat in pursuit of the cricket.*** *Time to intercept and eat the cricket was recorded. If the rat caught the cricket, it was given sufficient time to finish eating the cricket before being removed from the arena. Rats that did not catch the cricket were given a max latency of 120 s.*

Testing sessions took place in the second week. Rats were given live-cricket-hunting trials for 5 consecutive days. Each day consisted of 4 trials of maximum 2 min duration. For each trial, the rat was first placed in the starting cylinder with the lid on in one of the four corners of the area (the same sequence of positions was used each day for each rat) for 15 s, during which a live cricket was released in the arena in the corner opposite to the rat. The starting cylinder was then removed, and the trial began. The rat was given 2 min to catch the cricket (Fig 10.B/C). The rat was removed from the arena when the cricket was eaten or if the 2 min trial duration elapsed (whichever happens first). Crickets were not reused. The arena was thoroughly cleaned of all debris and wiped with water between trials. Rats were held in the holding bucket between trials, the intertrial interval was 1 to 2 min. On day 5 of testing, pseudorandom locations were used for the positioning of the cricket at the start of the trial, *i.e.* not always opposite to the rat.

Analyses: Latency to first intercept (nose within whisking distance), catch (cricket in mouth or paws) and to start to eat the cricket were scored manually either at the time of the experiment watching the live video stream or later using the recorded videos, in case rats captured and consumed the crickets too rapidly for reliable live scoring.

Locomotor activity (e.g., distance moved and time in arena zones) was tracked using ANYmaze software to generate distance moved and % time in different zones of the arena (e.g. center and outer periphery during 10 min habituation trials when cricket was not present).

11	JUVENILE PAIRED PLAY

Apparatus: Four horizontally interconnected modules of the Habitat were used as the home-cage environment for the duration of the experiment (Fig. 11A). Each module had an infrared CCTV camera placed outside that recorded continuously. Lights turned on 02:30 and off on 14:30. Water was provided *ad libitum*. Food was provided in excess and distributed on the floor of the modules. Food was regularly replenished every Monday and Friday and was topped up as necessary. Tubes provided access between modules and solid transparent sliding doors could be slotted between modules to close off access. Enrichment objects were provided (red crawl ball, plastic ladders and yellow house). USV recording microphones (Avisoft Bioacoustics) were positioned over each module.

Procedure: A pair of juvenile rats (P23-28, usually littermates, weight-matched, same sex, of same or different genotype) were housed together in one row of the Habitat for a total of 13 days.

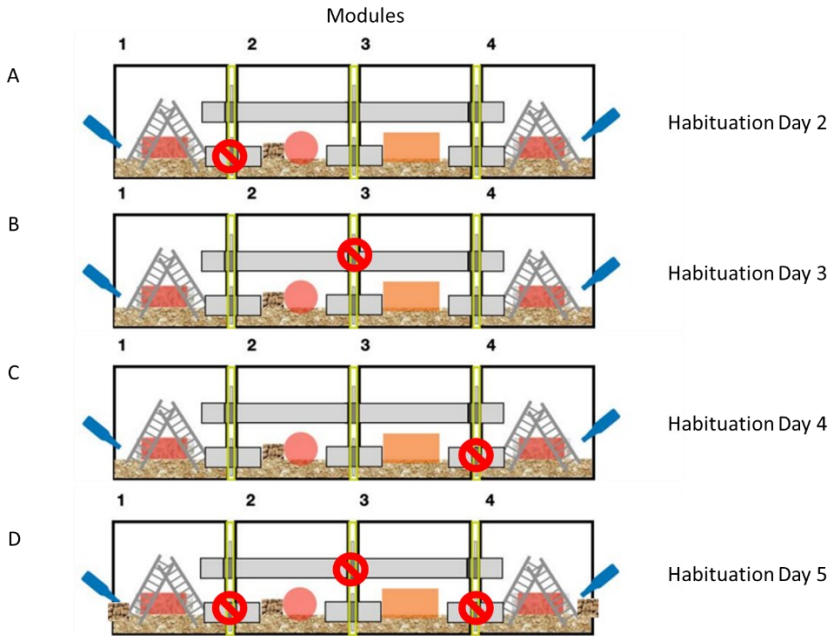


Figure 11.1. Juvenile Paired Play Habituation Schematics *A) Habituation Day 2 where bottom left door was blocked in the Habitat, rats have access to the entire row; B) Habituation Day 3 where top middle door was blocked in the Habitat, rats have access to the entire row; C) Habituation Day 4 where bottom right door was blocked in the Habitat, rats have access to the entire row; D) Habituation Day 5 where bottom left and right doors and top middle door were all blocked. Each rat had access to only 1 module (module 1 or 4) and half of the top tunnel connecting 1 and 4 (blocked in the middle). This habituation to isolation lasted 30 minutes around lights-off period*

Rats were first habituated to a row of modules in the habitat as a pair (Day 1). Rats then received three days of door habituation (Day 2-4) and one day of isolation habituation (Day 5). We used peanut butter on days 2-5 as a positive association during habituation, to counter the possible negative effect of noises and configuration changes of the door manipulation. A blob of peanut butter was wiped on the inside of the wall containing the water bottle (module 1 and 4) after door insertion or removal.

On days 2, 3 and 4 doors were inserted between modules 1 and 2 (bottom left, Fig 11,1A), then between modules 2 and 3 (top middle, Fig 11,1B), and lastly between 3 and 4 (bottom right, Fig 11,1C). For days 2-4, the doors were inserted before the lights went off (e.g. 14:25) and remained in position for 24 h. After 24 h, the door was removed and immediately placed in an alternate location, always paired with the provision of peanut butter in the outer modules. This protocol encouraged the rats to explore alternative routes around the habitat and get acclimatised to the doors sliding in/out before the isolation stage.

On day 5, all three aforementioned doors were blocked (small lower tubes between module 1 and 2, and 3 and 4, and between the upper tunnel between module 2 and 3, see Fig 11.1 D) and rat pairs were separated for 30 minutes, this served as habituation to isolation. -The rat pairs were isolated using the sliding doors to separate them into either module 1 or 4 beginning approximately 15 min before lights-off-. Each rat only has access to 1 module and one half of the top middle tunnel connection module 1 and 4 (blocked in the middle) during this period. The isolation was achieved by the use of peanut butter wiped on both ends of the row of modules, which encouraged rats to separate. Usually, inserting and removing doors as animals move across the modules would be sufficient to isolate a pair. Occasionally, if the pair cannot be separated by door placements only, and light-off period was approaching, we would physically retrieve a rat to separate them. After 30 minutes of separation (ideally 15 minutes before and after lights-out), doors were removed, and rats could reunite and access the entire row.

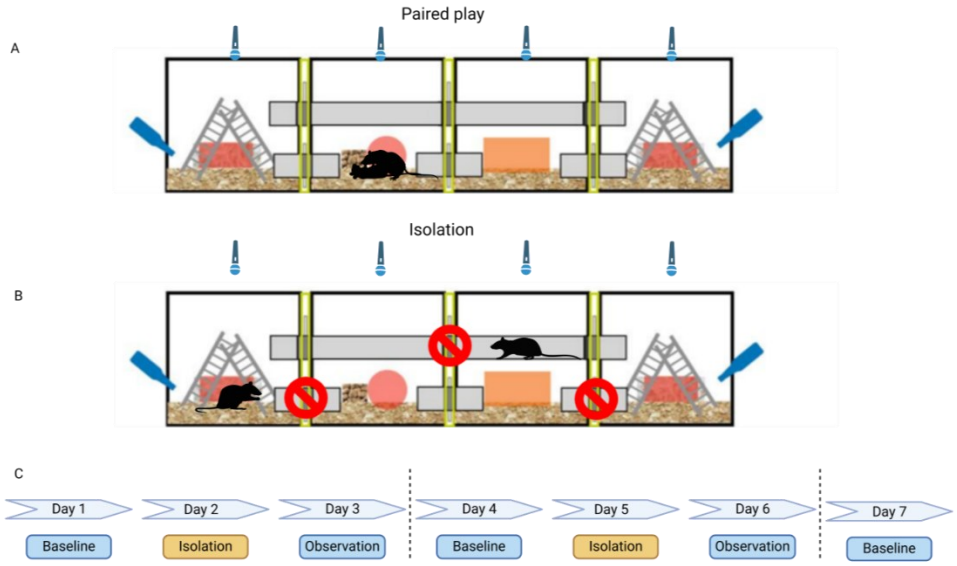


Figure 11.2. Juvenile Paired Play Schematics *A) Paired play set-up with two rats in a row of 4 modules in the Habitat; B) Isolation set-up with one rat on each side, with tunnels between the two halves blocked; C) Testing schedule*

Testing, in which rats were twice separated and reunited (which typically results in high levels of rebound play following reunition), began after the 4 days of door of acclimation, when rats were P33-40 of age. The method of separation was the same as described for day 5, isolation habituation. On the first testing day the pair had access to all 4 modules together, and their behaviour was monitored for 2 h after lights-off (baseline, Fig. 11.2.A). Rats were isolated for 24 h on Testing Day 2 (Fig.11.2. B). On Day 3, animals were reintroduced by removing the sliding doors between the modules. Observations were made for 2 hours after lights-off for post-isolation play. The same 3-day schedule (baseline-isolation-reintroduction) was repeated one more time, with an extra day of observation of paired behaviours on Day 7 (Fig. 11.2C).

Analyses: Latency, duration and bouts to social interaction were measured at each baseline and post-isolation stages for 2 h after lights were off.
Behaviour was scored using BORIS to measure when and where play behaviour took place. Play behaviours are defined as reciprocal interactions that start with physical contact and end when the playful behaviour is not reciprocated within 1 s. Hops and running are not play unless physical contact has been made and reciprocated. Examples include but are not limited to nape attack, rotated defence, pin, facing defence, mutual upright, evasion, chase, locomotion play, object-directed play, and tail tug, etc.
A passive pin is always counted as play. A chase that is not followed by another play bout does not count as play. Inter-rater reliability is checked by plotting the scoring of the behaviours to make sure no clear and distinct bouts of plays missed by any scorers.

12	ONE TRIAL ADULT SOCIAL INTERACTION

Apparatus: The arena (60x60 cm, Kanya UK) had white laminated wood walls (60 cm) and a white laminated wood floor. The light intensity was uniformly maintained at ~20 lx. An overhead camera recorded all trials, and ultrasonic vocalisations were recorded in the test trial using a fixed microphone (Petterson 500) suspended approx. 1.5 m above the arena. The arena was cleaned with 70% ethanol between rats. The experimenter watched the trials on a screen from a separate room and was ready to intervene if any aggressive behaviour was seen (wrestling and audible vocalisations, which was very rare).

Procedures: rats were first placed in the arena in cage groups for 10 min twice on Day 1. Test rats and the rats they were to be paired with (stimulus rats, which were unfamiliar same sex and strain and within 1-3 wks of age of the test rats) were also placed in cage groups into the arena twice for 10 min, with approximately 1-2 hr between sessions. On Day 2, all rats were placed individually into the arena for 10 min.

On Day 3 all rats received a test trial of 15 min duration in which the rat was placed into the arena for 5 min, and then an unfamiliar same-sex rat was introduced to the arena into the opposite corner to which the test rat was. After 10 min together, the test rat was removed from arena first and placed into a clean holding cage to avoid transferring any odours to his cage mates. Then the stimulus rat was then removed and placed back into its home cage. Stimulus rats could be used up to two times with different test rats within a day, with at least 1 hr between trials. Stimulus rats were not used in more than 3 experiments.

Analyses: Videos were scored using BORIS software to manually quantify the time that the test rat spent engaged in social exploration of the stimulus rat (nose within whisking distance, grooming, climbing on, placing paws on, boxing with, following closely or pinning the stimulus rat). Activity was tracked using ANYmaze to generate distance moved and % time in different zones of the arena (e.g. center and outer periphery during the habituation trials when the other rat was not present).

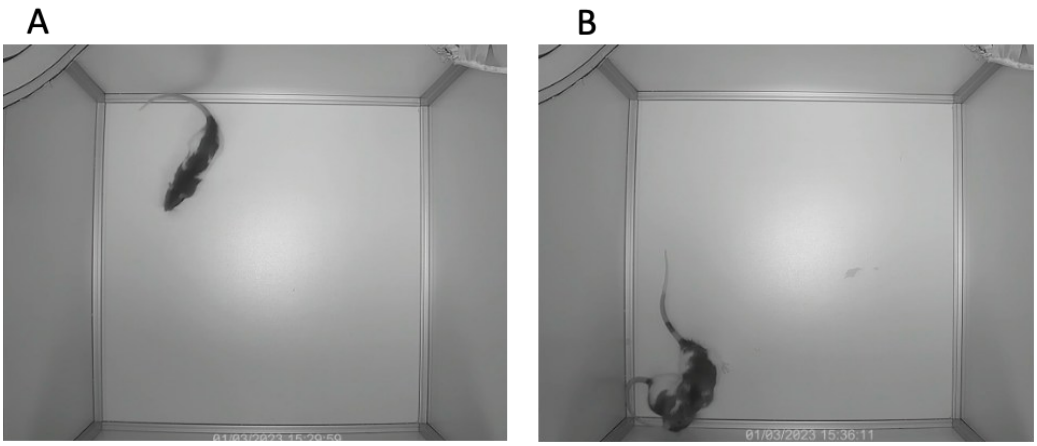


Figure 12. One trial social interaction task schematic during test trial. *A) Test rat was put in the arena alone for 5 min; B) An unfamiliar rat was then added to the arena for a further 10 min. All social exploration was manually scored (including licking, pinning, sniffing/nose within whisking distance, close following/contact with body).*