Method Article

Using the pup retrieval instinct as reinforcement for efficient auditory learning in mice

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A B S T R A C T

There is growing interest in the mechanisms for natural sensory learning in pro-social contexts. Studies using a maternal model of social behavior in the mouse have provided new insight into the auditory processing of behaviorally relevant pup vocalizations, which are used as communication signals to elicit pup retrieval behavior by adult females. Whether neural and behavioral plasticity in response to these vocalizations reflect auditory associative learning linking the sounds to pups, versus simply a change in maternal responsiveness to evolved vocal signals, remains an open question. Here we describe a T-maze paradigm to track auditory learning as we pair an initially neutral, non-ethological stimulus with delivery of a pup for retrieval, which is intrinsically reinforcing for rodents.

- Training is rapid and completely appetitive.
- Over a period of 7 × 50-minute daily training sessions, animals increasingly use the sound to guide their arm choice for pup retrieval, with an increase in performance from chance to an average of ~80% on day 7.
- This pairing method establishes a newly-formed sensory association using a natural maternal behavioral response, and lays a solid foundation for studies into the neurochemical and circuit mechanisms that mediate auditory associative learning in natural social contexts.

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A R T I C L E  I N F O
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Specifications table

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| More specific subject area | Sensory Learning |
| Method name | T-maze Paradigm for Auditory Learning with Pup Reinforcement |
| Name and reference of original method | [1]. Becoming a better parent: mice learn sounds that improve a stereotyped maternal behavior. *Hormones and Behavior* |
| Resource availability | https://github.com/rcbliu |

### Maze design

All our studies are conducted inside an 8 × 10 ft. double-wall anechoic chamber (IAC) with red cellophane foil covering all the lights so that our experiments are done under dim red lighting conditions, where light sensitivity is low in mice [3]. We use a basic T-maze design with a nest area (5.25 inches × 5.25 inches) on the base of the T and two equidistant arms (4.25 inches wide, inside width, with 0.75 inches tall triangular walls) with speakers on each side (Fig. 1). It is important in the design of the apparatus to have a clear decision point, which in this case is at the intersection of the T, in order for the experimenter to detect when the animal is making its choice. The length of our maze is 32 inches and the width is 24 inches (each arm is 12 inches). The maze is elevated from the ground 11.8 inches and is made out of wood sealed in a washable black paint. Speakers are placed on each end of the arm (approximately 10 inches away from each arm's edge), not parallel to the arms, but instead angled both vertically and horizontally to point down towards the decision point (see inset), so that a perpendicular line from the center of the speaker crosses the T at about 2 inches above the T-maze floor. The ends of the arms are open, i.e. no wall. We use a remote control button as a wired switch to turn on our stimulus, which is played randomly from one side or the other on each trial. We pair stimulus onset with a red light emitting diode (LED) that either blinks or stays constant, depending on the sound delivery side. Since our stimulus is ultrasonic, the elevated LED, which is shielded by cardboard from the subject’s view, allows the experimenter to know where to deliver a pup on a given trial in order to match the sound delivery side.

![Fig. 1. T-maze apparatus for pairing novel sounds with reinforcement by pup retrieval. Inset shows photo of the actual set up with the positioning of the left speaker relative to the edge of the left arm.](image-url)
Auditory stimulus

We use a custom MATLAB (Mathworks, Natick, MA) script to generate a daily lookup table of 100 trial-by-trial parameters. It contains two parameters that vary from trial to trial: the side or arm from which the sound will be delivered, and a scaling factor to control relative sound volume. The side of sound delivery is chosen randomly with 50% chance on either side, with the constraint that no three consecutive trials can be delivered from the same side. The volume is roved randomly from trial to trial over a range of 13 dB.

Stimuli are generated and delivered by a RX6 multifunction processor and the OpenEx software suite (Tucker Davis Technologies, Alachua, FL) that interfaces a PC computer with the RX6 (Fig. 2). The RX6 outputs signals at a sample rate of 223,214 Samples/second. Before a training session starts, the lookup table is loaded into the OpenEx Controller. During the training session, the experimenter pushes the remote button in the training booth to initiate a trial. The remote button connects through a PP24 patch panel to the RX6 to trigger RPvdsEx code running on the RX6. This generates a stream of Gaussian white noise, which is filtered by a Butterworth filter (center frequency = 40 kHz, bandwidth = 33 kHz) and then multiplied element-wise in time by a 5 Hz sinusoidal amplitude envelope. A software switch delivers the sound to one of the two RX6 digital-to-analog outputs at a particular overall amplitude, based on the lookup table's parameters. The output is passed to the corresponding speaker (Pioneer, model PT-R4) sitting on each side of the T-maze arm. In parallel with the sound delivery, the RX6 also outputs a constant TTL high pulse when the sound is sent to the “left” speaker, or a sequence of 3 Hz TTL pulses when it is sent to the “right” speaker. These drive the red LED that is only visible to the experimenter and the recording camera. When the experimenter pushes the remote button again, the playback and LED are shut off, and the next trial does not begin until the remote button is depressed again.

Animals

All animal procedures were approved by the Emory University Institutional Animal Care and Use Committee. Subjects are adult female mice that are naive to pups (except from when they were pups themselves), have pup-care experience (co-carers), or lactating or post-weaning mothers. All are able
to learn. All animals are over 8 weeks old when they begin the training. Pups used for reinforcement are provided by a foster cage, and should be between postnatal day 3 to postnatal day 7 in age.

Set up

We cover the T-maze surface with Alpha-dri bedding, which is soft and produces less noise when animals walk over it. Bedding should be changed, and the T-maze should be wiped down with a disinfectant (e.g. Virkon or MB-10), between each animal run on the T-maze. Each training session is recorded with a video camera positioned above the center of the T-maze in order to score the behavior offline. The sequence below outlines in detail the steps the experimenter follows on each day of habituation and training.

Day 1: habituation (10 min)

Transport the subject from its home cage to the maze in a glass beaker to reduce stress. Place the beaker horizontally on the base of the T-maze near the nest area and let the animal leave to explore the maze freely, then remove the beaker. Since the maze is inside an anechoic chamber, it is important for the animals to get habituated to the unusual absence of sound [2]. Leave the anechoic chamber door slightly open for the first 5 min, then slowly close the door. Sit next to the maze without making any fast, sudden movements that could alarm the animal. Instead, make small movements that would be typical of what the mouse will encounter during training (e.g. the swivel of a chair), so the animal acclimates to the experimenter. If the animal freezes most of the time, it is important to remain calm and not make sudden movements that cause the subject to try to flee. Animals typically unfreeze and calm down after a few minutes on the maze, as long as the experimenter is quiet and does not move too much. When the animal starts moving, the experimenter can also make small movements. After 10 min, introduce the beaker and lead the animal inside with your hand. Transport the animal back to its home cage.

Day 2: habituation (10 min)

Repeat the procedures from Day 1 Habituation. Close the anechoic chamber door immediately for all 10 min. The animal should feel more comfortable and explore the maze freely on Day 2. Continue making small movements and halfway through, the experimenter may introduce a gloved hand slowly to help the subject acclimate to the experimenter's scent. After 10 min, use the beaker to transport the animal back to its home cage.

Day 3: habituation (10 min)

Transport the animal from its home cage to the maze in a beaker. The animal should feel comfortable and explore the maze freely. The experimenter should introduce her hand a few times so the subject can smell it and get used to a person reaching over the maze. The experimenter can also make bigger movements by this stage, such as standing up or opening and closing the door to habituate the animal to noise and movement. After 10 min use the beaker to transport the animal back to its home cage.

Day 4: training session 1 (max 50 min)

Before introducing the subject to the maze, verify that the remote button and speakers are working properly by checking sound levels with a bat detector (Mini-3, Ultrasound Advice). Using a small glass beaker with new bedding, transport two pups from the foster cage to the nest area of the maze. Leave the small beaker inside the room to use during training. Transport the subject from its home cage to the maze in a larger glass beaker, and let the animal habituate to the maze environment for 10 min with pups in the nest area. After 10 min, take one of the pups from the nest and wait for the subject
Fig. 3. Timeline for training. (A) Subjects are habituated to the maze environment for 3 days. (B) Daily training sessions of up to 100 trials each where subject make a choice to the Correct (green) or Wrong (red) arm, based on whether the sound plays from that side. (C) By 7 days of training, subjects learn to follow the sound correctly for a pup reward. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

to be near the nest area. While the subject watches, move the pup at the level of the bedding 5–10 inches away from the nest, which typically induces a retrieval behavior. It is not uncommon for ~10–20% of subjects to ignore pups and not retrieve at all on this first day, especially if working with animals that are naïve to pups. After the subject retrieves pups from the main arm to the nest a few times, move the pup all the way to the end of one arm and let the subject retrieve it back to nest. Repeat with the other arm. During each of these outings, as the subject is retrieving one of the pups back to the nest, remove the other pup from the nest and place it in the small beaker in the experimenter’s hands so that it can be used for the next retrieval. As soon as the second pup is returned to the nest area, press the remote button to initiate the sound playback. This starts the first trial and the training session.

In the beginning, it is normal for the subject to stay in the nest and ignore the sound. Eventually, the subject will leave the nest area and approach a side (Fig. 3). The concurrent LED indicates to the experimenter only which speaker the sound is coming from, so that the experimenter knows where to deliver a pup for retrieval. Importantly, no pup is placed at the end of the sound playback arm at this time, so that the physical pup cannot serve as a cue for which arm to choose. The experimenter infers the subject’s choice by observing when 1/3 of its body enters into one of the arms (past the intersection with the base of the T). If the subject makes an incorrect choice, meaning that it enters the side where no sound is playing, then the experimenter simply waits for the subject to go to the other arm. No punishment is necessary, though this delay in reaching a pup may act as a natural “time-out.” As soon as the subject makes the correct choice to go down the arm where the sound is playing, the experimenter places a pup near the end of the arm so that the subject can reach and retrieve it to the nest. It is important to give this pup “reward” as soon as the subject makes the correct choice. If the reward is delayed so that it is not paired with the animal’s decision, then it is typically harder for the animal to create the association between its choice to follow the sound and the pup reward. As soon as the animal retrieves the pup, the experimenter turns off the sound by pressing the remote button again, and waits for animal to return to the nest with its retrieved pup
for the next trial to start. Occasionally, the animal will not retrieve the pup all the way back to the nest, in which case the experimenter should move the pup back to the nest and avoid starting the next trial until both the pup and the subject are back in the nest.

As the subject is retrieving one of the pups back to the nest, remove the other pup from the nest and place it in the small beaker in the experimenter’s hands so that it is ready to be delivered in the next trial. As soon as the subject returns to the nest and drops the pup, press the remote button for a new trial and repeat.

After the subject completes 100 trials or reaches the 50 min time limit, transport the animal back to its home cage. The OpenEx project can be programmed so that after 100 trials the light does not turn on, indicating to the experimenter that the training session is over.

Days 5–10: Training sessions 2–7 (max 50 min each)
Repeat Training session 1 with no changes. As the foster pups age above P7, younger pups from another foster cage may be used; older pups become larger and more self-mobile, which can slow the training process. Subjects typically begin to complete trials faster and eventually follow the sound for a pup reward. While there is some variability across experimenters, in our experience, subjects start to follow the sound around Training sessions 3–4, and the time it takes for them to complete 100 trials decreases each day.

Clean up
Pour dirty bedding in an empty cage, wipe down maze with disinfectant, and vacuum remaining bedding around the maze.

Scoring
We manually score the videos using the Observer XT software (Noldus), but any other behavioral scoring software may be used. We quantify the extent to which animals use the auditory cue to guide them towards a pup reward by scoring each trial as “Correct” if the animal entered the arm from which the stimulus was playing (based on the LED in the video), or “Wrong” if they entered the opposite arm. We call this the “Auditory Strategy.” We also score the trials using a “Location Strategy,” wherein we would score the trial as “Correct” if the subject entered the arm from which a pup was delivered on the previous trial, and “Wrong” if it entered the opposite arm. Most subjects innately use this strategy in the beginning of the training period to guide their choice, and eventually they learn to use the sound stimulus instead of their spatial memory [1]. Note that on any given trial, both strategies can be scored as “Correct.” Two additional events scored are the onset of the stimulus (when the light turns on) and the time at which the subject makes a choice. Scoring can be done offline and blind to the animal, training session, etc., and can be carried out by more than one scorer.

Method validation
In a separate cohort of n = 4 subjects from those presented in Dunlap et al. [1] (companion paper), we confirmed that on day 1 of training, most animals use the Location Strategy on ~80% of trials and the Auditory Strategy on ~50% of trials (chance level). After training subjects for 7 days, subjects increase their use of the Auditory Strategy to close to ~80% on average (Fig. 4), while their use of the Location Strategy decreases to chance. This confirms that animals are learning to use the auditory cue to guide them towards a pup reward.

Recommendations/additional information
We have found the training paradigm in our hands to be quite robust across different experimenters. Nevertheless, some factors can affect performance as well as the motivation to act maternally and perform in the apparatus. For example, if subjects are pharmacologically or surgically manipulated, they can move more slowly and take longer to make a choice. Maternal responsiveness may also be affected in this case, especially if the subject is exposed to a treatment (e.g. injection)
Fig. 4. Daily performance using the Auditory Strategy improves with training. (A) Trial-by-trial performance of an example subject that learned across 7 daily sessions (each row). Red = Wrong; Green = Correct based on the Auditory Strategy. (B) Group data from n = 4 subjects, all of which learned to use the Auditory Strategy. Performance in the first 10 trials of each session agreed well with overall performance for the entire session. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

Fig. 5. Y-maze apparatus built inside a rat-sized cage for pairing novel sounds with reinforcement by pup retrieval. This was less effective for training animals as compared to the original T-maze design.

before training. In such cases, we have found it useful to introduce pups into the nest area earlier, such as halfway through the last day of habituation, in order to sensitize subjects to pups for longer. This can help speed up the initial retrieval process on the next day, and ensure animals will retrieve. Furthermore, the timing of when the pup is given as a reward is crucial. We find that if the pup reward is not paired with the animal’s choice (e.g. delayed delivery of the pup), then it is harder for the subject to make the association and be successful in the Auditory Strategy. We have also seen that the more handling we do with a subject as it habituates to the paradigm, and the more comfortable a subject seems to be with the experimenter, the better its chances of learning.

Our experience also suggests that some physical parameters about our behavioral apparatus are crucial for the animals to learn. For instance, the maze must have a clear decision point. We have tried to use a smaller Y-maze created inside a rat-sized cage to perform the training. Although around a
third of subjects could learn to use an Auditory Strategy, performance was poorer than in the T-maze and training took longer. One reason may be that the decision point in that specific maze (Fig. 5) was too ambiguous. Its compact design, which was initially preferred in order to run cages in parallel, may have decreased the cost to the subject of making a wrong choice. Related to this, the distance from the nest to the decision point was likely too short, preventing the animal from carefully using the auditory information to base its decision. In the T-maze, animals have ~30 inches of travel before they make their choice, but the distance between the nest area and the decision point in the Y-maze was only 6 inches.

Declaration of Competing Interest

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.101051.

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