Video Article

Whisker-signaled Eyeblink Classical Conditioning in Head-fixed Mice

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URL: http://www.jove.com/video/53310
DOI: doi:10.3791/53310

Keywords: Behavior, Issue 109, trace conditioning, cylindrical treadmill, learning and memory, hippocampus, cerebellum, whisker barrels

Date Published: 3/30/2016

Citation: Lin, C., Disterhoft, J., Weiss, C. Whisker-signaled Eyeblink Classical Conditioning in Head-fixed Mice. *J. Vis. Exp.* (109), e53310, doi:10.3791/53310 (2016).

Abstract

Eyeblink conditioning is a common paradigm for investigating the neural mechanisms underlying learning and memory. To better utilize the extensive repertoire of scientific techniques available to study learning and memory at the cellular level, it is ideal to have a stable cranial platform. Because mice do not readily tolerate restraint, they are usually trained while moving about freely in a chamber. Conditioned stimulus (CS) and unconditioned stimulus (US) information are delivered and eyeblink responses recorded via a tether connected to the mouse's head. In the head-fixed apparatus presented here, mice are allowed to run as they desire while their heads are secured to facilitate experimentation. Reliable conditioning of the eyeblink response is obtained with this training apparatus, which allows for the delivery of whisker stimulation as the CS, a periorbital electrical shock as the US, and analysis of electromyographic (EMG) activity from the eyelid to detect blink responses.

Video Link

The video component of this article can be found at http://www.jove.com/video/53310/

Introduction

Eyeblink conditioning is a form of Pavlovian conditioning and a model system for investigating the neural mechanisms of associative learning and memory. It has been investigated in various species, including humans, rabbits, cats, rats, and mice. The paradigm involves the presentation of two paired stimuli: a neutral conditioned stimulus (CS; *e.g.*, a tone, a flash of light, or whisker stimulation), and a salient unconditioned stimulus (US; *e.g.*, an air puff to the eye, or periorbital shock). The US elicits an unconditioned, reflexive eyeblink response (*i.e.*, UR). Eventually, after several presentations of the paired CS-US, the subject learns to associate the CS with the US. This learning manifests itself in the form of a conditioned response (CR), an eyeblink elicited by the CS alone that precedes the presentation of the US.

Eyeblink conditioning in the trace form includes a stimulus-free interval of a few hundred milliseconds that separates the CS and the US (**Figure 1**). Trace conditioning is a form of declarative learning since it requires awareness of the stimulus contingencies[1]. The temporal gap requires the animal to keep a neural 'trace' of the CS in forebrain regions such as the hippocampus in order for the US and the CS to become associated[1-6]. Along with the forebrain regions, trace conditioning is also dependent on the cerebellum[7].

Eyeblink conditioning is, therefore, a useful paradigm for the investigation of the multiple facets of memory, including acquisition, consolidation, and retrieval. During eyeblink conditioning, a control group of animals is presented with unpaired stimuli in random order to test for pseudoconditioning or sensitized responses to the CS that may be caused by US presentation alone rather than a learned CS-US association.

A commonly used apparatus for the investigation of eyeblink conditioning in rodents is a chamber in which the rodents are allowed to move about freely during the training process[8-10]. With this type of apparatus, a tether is normally attached to a headpiece that is affixed to the rodent's skull. The tether allows for the delivery of the US (and sometimes the CS) and for transmitting the animal's response to those stimuli (*i.e.,* the eyeblink response)[10]. The tether itself may be modified based on the type of stimuli delivered and how the eyeblink response is recorded.

The reason for using "freely-moving" tethered mice for eyeblink conditioning is that mice struggle against restraint. Though other species may be more amenable to restraint, the major advantage of using mice in eyeblink conditioning experiments is that the majority of available genetically modified mutant strains are mouse strains. In addition to struggling, complete restraint of mice results in acute distress. A head-fixed mouse preparation that minimizes stress would increase the physiological information that can be obtained during eyeblink conditioning. For example, this system would allow imaging of cortical neurons with 2-photon microscopy[11].

Head-fixed preparations have been used in previous experiments for optical imaging of the cortex through removable cranial implants, *in vivo* electrophysiological recordings of the rodent brain with tetrode arrays, two-photon calcium imaging, and also as a platform for eyeblink conditioning in mice[11-16].

In the head-fixed system, reliable stimulation and recordings are ensured without complete restraint of the mouse (**Figure 2**). A headpiece like the one used in the freely moving system is affixed to the mouse's skull. During training, the headpiece is affixed to a connector that is attached to bars over a cylindrical treadmill in order to stabilize the rodent's head (**Figure 2A**). The cylindrical treadmill allows the mouse to rest comfortably, but if the mouse so wishes, also allows it to run or to walk. With the use of this system, mice can be trained with a whisker vibration

as the CS and a mild periorbital electrical shock as the US (**Figure 1**). The US is delivered through wires surgically placed underneath the skin lateral to the eye. The CS is delivered via a comb that is attached to a 2-layer rectangular bending actuator (**Figure 2B**). The comb and bending actuator are then attached to a magnetic base that is moved to the proper position during training and is adjusted for optimal delivery for each individual animal. The comb is positioned to straddle the selected whiskers. During delivery of the CS, a signal is sent to the bending actuator that displaces the comb and leads to vibration of the whiskers[17].

Other stimuli such as a tone or a flash of light have been used as effective conditioned stimuli in mice in the past[16,18,19]. The reason whisker stimulation is chosen for the CS in this experimental paradigm is the dependence of murine animals on their vibrissae for somatosensory information input during exploration. Whisker stimulation has been shown to be a reliable and effective CS[20]. Furthermore, given the well-established and organized cortical substrate of the vibrissae system (*i.e.*, the barrel cortex), whisker stimulation as the CS provides an elegant tool for mapping cortical changes and plasticity associated with learning eyeblink conditioning[20,21]. A head-fixed system allows for the precise stimulation of selected whiskers to compare responses between stimulated neurons and neurons receiving inputs from non-stimulated whiskers. Finally, many strains of mice exhibit age-related hearing loss as relatively young adults[22], and eyelid closure during the conditioned blink alters a visual CS (although a visual CS does ameliorate issues with startle responses[16]). Whisker stimulation is not affected by either of these complications.

Presented here are unique and important modifications upon other head-fixed preparations for eyeblink conditioning, including methods for CS and US delivery, and the acquisition of the eyeblink response. The reliability of this apparatus and the training paradigm in eyeblink conditioning is demonstrated by learning curves from conditioned mice and a relatively flat learning curve from pseudoconditioned control animals (**Figure 7A**).

Protocol

All procedures involving mice were performed in accordance with protocols approved by Northwestern University's Institutional Animal Care and Use Committee based on guidelines issued by the National Institute of Health.

1. The Cylinder (Figure 2A)

1. Construct the cylinder as described by Chettih *et al.* and Heiney *et al.* from a long foam cylinder[14-15]. Cut a 10 cm length of cylinder and drill a hole through the center to fit the axle, a metal rod 12.7 mm (0.5 in.) in diameter. Mount the cylinder with its axle onto a metal optical breadboard as described by Heiney *et al.* or another support (*e.g.,* Plexiglas)[15].
2. Erect two vertical metal rods of 12.7 mm in diameter on either side of the cylinder.
3. Attach two right-angle mounts onto the metal rods.
4. Secure two more rods (5 cm long, 12.7 mm in diameter) through the right-angle mounts. Bevel the ends of these rods and level their ends above the center of the cylinder (**Figure 2G**).
5. Drill and tap a hole through the leveled ends of the rods to fit two 4-40 machine screws that will hold the wings of the connector in place (**Figure 2G**).

2. Assembling the Whisker Stimulation System (Figure 2B)

1. Cut a 10-tooth segment of comb from a regular hair comb.
2. Cut a slit about 2-3 mm wide and about 5 mm deep on the top part of the comb to accept a 2-layer rectangular bending actuator.
3. Solder one wire to each surface of the bending actuator. Use the solder and flux provided by the manufacturer. Cover the actuator and solder with electrical tape to protect the mouse and user from the electrical current.
4. Slip the actuator strip into the slit cut into the comb so that the bending actuator strip sits perpendicular to the plane of the comb. Angle the comb so that it is tilted at 45° and more naturally follows the curve of the mouse's face.
5. Secure the end of the actuator strip to the top of the comb with quick-setting epoxy.
   Note: The wires soldered to the bending actuator strip should be connected to a rheostat that delivers 40 volts to the actuator. Previous experiments have shown that 40 volts is effective for conditioning but does not evoke any startle responses.
6. Place the bending actuator and the comb on a moveable mount. A pipette holder attached to a magnetic base as normally used for *in vitro* electrophysiology rigs works well.

3. Assembling the Connector that Mounts to Rods above the Cylinder (Figure 2C, 2E)

Note: The connector is a 3-D printed 7-hole strip modeled from the Amphenol 221 series nylon strip used for the tether by Weiss and Disterhoft and Galvez *et al.*[10,17]

1. Tap the first hole in the strip for a 0-80 x-1" machine screw. This will serve as a locking screw to anchor the headpiece to the connector. Affix a 0-80 nut to the head of the locking screw with cyanoacrylic glue in order to facilitate turning the screw by hand.
2. Leave one hole empty after the tapped hole (to allow room for the 0-80 machine nut) and push five gold-plated relia-tac sockets through the remaining five holes.
3. Strip the coating off five long wires (wires obtained from an Ethernet cable) and solder to the ends of the sockets.
   Note: The first two wires will be used to record the electromyographic (EMG) signals that will detect an eyeblink response following signal filtering and amplification from an amplifier. The second two will deliver the shock signal from a stimulus isolator. The last wire will serve as a ground connection. These wires will be connected to the systems used for the timed CS-US delivery.
4. Ensure electrical connectivity between the wires and the sockets with a multimeter. Put the multimeter on the continuity setting and hold one of the probes to the pin and the other probe to the stripped ends of the wire. The pins and the wires are electrically connected when the resistance is low and the multimeter emits a tone.

1. Alternatively, if the multimeter does not have a continuity setting, measure the resistance between the pins and the wires. There is electrical continuity if there is very little resistance measured.

5. Prepare the wings of the connector from two TO-220 style transistors. Cut off the emitter, base, and collector leads of the transistor and level the surface so it can be affixed to the connector.
6. Cut the edge of the mounting tab to breach the hole so the tab can slip underneath the head of the screw.
7. Epoxy the transistors to the connector. The hole in the mounting tab will be used to secure the connector to the rods above the cylinder.
8. Apply epoxy to the base of the wires protruding from the sockets and allow the epoxy to dry. The epoxy will insulate and protect the connections.

4. Preparing the Headpiece (Figure 2D, 2E)

Note: The headpiece strip is a 3-D printed 7-hole strip modeled from the Amphenol 221 series nylon strip used for the headpiece by Weiss and Disterhoft and Galvez *et al.*[10,17]. This piece is no longer manufactured commercially. The printer file can be downloaded from this journal's website.

1. Tap the first hole in the strip for a 0-80 x 1" machine screw and leave one hole empty after the first hole.
2. Push five gold-plated pins through the bottom of the remaining five holes (through the narrower ends). Use a vise to facilitate pushing the pins into the strip evenly.
3. Using a thermal stripper, remove about 0.5 cm of polyimide coating off the end of a stainless steel wire (0.005 in. diameter), solder the stripped end of the wire to the opening of one of the pins and cut the wire to 0.6-0.7 cm.
4. Strip about 0.2 cm from the end of the wire to allow current to pass to the animal.
5. Repeat steps 4.3-4.4 for the remaining three wires.
   Note:The first two wires will record EMG responses while the second two will serve to deliver a shock to the animal.
6. As the last (fifth) wire will serve as a ground wire, cut about 5 cm of uncoated stainless steel wire (0.005 in. diameter), and solder to the remaining pin.
7. Ensure continuity between the wires and the pins with a multimeter. (In the case of the ground wire, which is uncoated, the multimeter probe can be placed anywhere along the wire.)

5. Surgical Preparation (Figure 2F)

1. Sterilize all surgical tools, including the headpiece. Sterilize the headpiece by soaking it in alcohol and then rinsing with sterile saline.
2. Anesthetize the mouse in an induction chamber with 3-4% vaporized isoflurane mixed with a flow rate of 1-2 L of oxygen per minute.
3. Ascertain that the animal is fully anesthetized with a toe pinch. The mouse is fully anesthetized when it does not respond reflexively to the toe pinch.
4. Once the animal has been fully anesthetized, inject a dose of buprenorphine hydrochloride as an analgesic (0.05–2 mg/kg, sc), shave the top of the animal's head, place it on a covered heating pad on the base of the stereotaxic frame, ascertain that the animal is fully anesthetized, and secure the head to the frame. Maintain flow of vaporized isoflurane to the animal, switching to 2% at a flow rate of 1-2 L of oxygen per minute. Apply a small amount of ophthalmic ointment to the corneas.
5. Disinfect the scalp with povidone-iodine and alcohol three time each, alternating between the two.
6. With a number 10 or 15 scalpel blade, make an incision along the midline of the scalp, exposing the skull from the front of the eyes to past the interparietal bone (~1.5-2 cm).
7. Hold back the flaps of the skin with micro clips. Place one clip above the eye, one along the middle of the rostral-caudal axis, and one at the back of the skull bilaterally (*i.e.,* use six clips in all). In order to prevent headpiece avulsion, expose as much of the skull as possible, including the sides and back. This will increase the surface area for the application of the adhesive luting cement.
8. Using the scalpel, scrape along the top of the skull to remove the periosteum and ensure a clean and dry working surface. Clean the top of the skull with 3% hydrogen peroxide three times.
9. Drill two holes into the skull with a size 34 inverted cone burr or a 1.6 mm engraving cutter drill bit to accept 00-90 screws (0.0625 in. long). The screws will provide the electrical ground connection. Place one hole in front of Bregma, and the other in front of Lambda, left of the midline when conditioning the right eye. Place one screw in each hole. Lower the screw 0.28 mm into the skull for each full turn; two full turns is sufficient.
10. Create several small divots of approximately 0.75 mm in diameter on the skull in order to increase the surface area and grip strength for the cement.
11. Take the completed headpiece and wind the ground wire in a figure-eight configuration around the two screws. Allow some slack (~1.5 cm between the headpiece and the screw) in the ground wire so the headpiece can be positioned properly later.
    Note: The figure eight usually ensures a good electrical connection between the wire and the ground screws. The wire can also be soldered to the screw to ensure an electrical connection.
12. Apply the adhesive luting cement. Follow the manufacturer's instructions for mixing the adhesive luting cement or mix 4 scoops of L-Powder mix, 8 drops of the base, and 2 drops of the catalyst in a cold ceramic mixing dish (ensure the temperature strip on the mixing dish is completely black.) The cold temperature extends the working time of the cement.
    1. Coat the skull and the screws liberally with the luting cement and allow the cement to dry. This should take only a few minutes.
13. After the cement has dried, position the headpiece vertically, with the pins standing up, above the skull. Hold the headpiece in place with a holder similar to the connector
    Note: See "3. Assembling the Connector"-The holder need only be a five-hole strip with gold-plated sockets in order to receive the pins of the headpiece. The holder is attached to an arm on the stereotaxic frame. The use of the holder and arm facilitates the positioning of the headpiece and the wires.

14. After the headpiece has been positioned, peel back the skin around the periorbital area and position the two shock wires to allow the 0.2 cm stripped end to rest underneath the skin and approximately 2-4 mm directly caudal to the eye. Do not allow the ends of the two wires to touch each other. Position the two EMG wires on the muscularis orbicularis oculi above the eye socket.
    1. If necessary, cut off the ends of the wires if they are too long and seem as though they may directly scratch the orbit and result in infection. If cutting off the ends of the wire, ensure that there is enough bare wire exposed. Alternatively, if the wires seem too long, bend the wire back at the base, where they extend out of the headpiece.
    2. Cement the base of the wires (*i.e.*, the end near the headpiece) in place on the skull with a small spread of the adhesive luting cement and allow to dry (use half of the portion described earlier).

15. Remove the micro clips and gently fold the flaps of skin back over the cement. Allow the skin to settle **naturally** to prevent tension on any part of the skin in order to avoid distortion of the eyelid, prevention of the eyeblink response, and distress to the animal.
16. Seal the exposed area with dental cement, covering everything from the incised skin to the edge of the top of the headpiece with cement. Take extra care to avoid dripping cement on the eyes or the pins of the headpiece. Partially cured cement can be smoothed and manipulated with a cotton swab that is dampened with dental cement solvent. Allow the cement to dry completely before removing the headpiece holder.
17. Allow the animal to recover from anesthesia on a warmed surface before replacing the animal back in its cage. Administer Metacam (1 mg/kg, SQ) and then place the animal back in its cage.
18. Allow the animal 5-7 days of recovery before testing or training. Maintain standard post-operative care to the animal according to your institution's guidelines. The animal should be checked at least once per day after it has recovered from surgery. Watch for signs of poor eating and/or drinking and listless behavior. If pain is suspected, provide Metacam every 24 hr (same dose as at the end of post-surgery) until alleviated. Lidocaine can be locally applied to the wound if the animal is scratching or shows signs of discomfort.

6. Placing the Mouse on the Cylinder and Training (Figure 2G)

1. To place the mouse onto the cylinder, restrain the mouse with a hand briefly by holding it by the tail with one hand and then grasping the mouse behind the shoulders with forefinger and thumb with the other. Wrap the remaining fingers around the mouse's abdomen and torso.
2. With the hand not restraining the mouse, attach the connector to the headpiece on the mouse's head and turn the locking screw.
3. Place the mouse gently on the cylinder and hold it in place while attaching the connector to the frame. Use the two screws to fasten the connector to the bars above the cylinder.
4. Once the connector has been secured to the cylinder frame, release the hold on the mouse. Give the mouse one day of two sessions of habituation to the cylinder. Allow each habituation session the same duration as each conditioning session.
    1. Record spontaneous blink rate during habituation and pre-expose the mice to the whisker vibration CS in order to minimize the occurrence of startle responses. Apply the CS as during an actual conditioning session, but without the electrical shock US.
    2. Apply the whisker stimulation CS by placing the Piezo system close (about 0.5 cm) to the conditioned side (the right side) of the mouse, while slipping the teeth of the comb over individual whiskers. Ensure that the same whiskers are stimulated day to day by placing the Piezo system in the same position from day to day.

5. Begin training the mouse on classical conditioning. Deliver two sessions of training per day for five days. Leave about two hours between each training session.
    1. To the conditioned group, deliver thirty trials per session of a 250 msec long whisker vibration CS (60 Hz) paired with a 100 msec long electrical pulse US (0.3 mA - 3 mA, delivered from a WPI A385R stimulus isolator). Separate the CS and the US with a 250 msec long stimulus-free trace interval to make the task dependent on the hippocampus (**Figure 1**)[23].
    2. Adjust the intensity of the electrical shock for each animal so that a blink is evoked. Separate trials with a random intertrial interval of 30-60 sec (average of 45 sec, total duration of 30 min per session.)
    3. Deliver thirty unpaired CS alone and US alone trials each (for a total of sixty trials) to the pseudoconditioned group, using the same stimulus parameters as stated for the conditioned group. Ensure that the CS alone and US alone trials are pseudorandomized such that no stimulus is presented more than twice in a row. Separate each trial with an average intertrial interval of 22.5 sec.

6. Deliver background noise at 65 dB throughout habituation and training in order to mask any noise of the vibration generated by the whisker stimulator.

Representative Results

8-10 week old male C57Bl6/J mice were trained on trace eyeblink conditioning on the head-fixed cylindrical treadmill apparatus. 8 mice were trained with paired CS-US presentations (conditioned group) and 9 mice were trained with unpaired CS and US presentations (pseudoconditioned group).

Example EMG recordings of a conditioned response from a conditioned mouse are shown in **Figures 3** and **4**. EMG recordings for each trial were rectified and integrated with a 10 msec time constant. **Figure 5** shows the rectified and integrated EMG response traces averaged across all trials for each of 10 sessions for both a conditioned (A) and a pseudoconditioned (B) mouse. The evolution of conditioned responses can be seen in **Figure 5A**, with responses getting larger closer to the onset of the US. This evolution is not seen in the pseudoconditioned responses in **Figure 5B**.

The evolution of well-timed, and thus, well-learned conditioned responses can also be seen in **Figure 6**. **Figure 6** shows the histograms of the time to the peak of the response following CS onset. There is an initial peak in the graph between 0 - 150 msec after CS onset. This initial peak can be seen as early as in session 1. With more training sessions, there is the development of a second peak in the graph between 400-500 msec amongst the conditioned animals, indicating the development of better-timed conditioned responses.

Figure 7A shows the averaged percent adaptive conditioned responses recorded from conditioned and pseudoconditioned mice. Adaptive conditioned responses are considered to have significant activity that is present at least 20 msec before US onset (*i.e.*, at least 4 SD greater than the mean activity present 250 msec before CS onset). Over the training sessions, conditioned mice showed gradual learning of the conditioning paradigm by exhibiting more conditioned adaptive responses with each training session so that conditioned mice exhibited significantly more adaptive conditioned responses than pseudoconditioned mice (groups, $F_{(1,15)}$=20.62, p<.0005; sessions, $F_{(1,9)}$=9.987, p<.0001; groups *sessions, $F_{(1,9)}$= 5.977, p<.0001). Note that the pseudoconditioned group typically exhibited pseudo CRs on less than 20% of the trials.

Figure 7B shows the averaged conditioned response area (the area under the curve of the rectified and integrated EMG response) for both conditioned and pseudoconditioned mice over the ten training sessions. Planned comparisons with a repeated measures ANOVA over the last four sessions (*i.e.,* sessions 7-10, once the conditioned group had reached a learning criterion of 60% CR) show a significant main effect of group, indicating that the CR area was larger in the conditioned group than in pseudoconditioned group post learning criterion (groups, $F_{(1,14)}$=5.733, p<.05; session, $F_{(1,3)}$=.486, *ns*; groups*session, $F_{(1,3)}$=.432, *ns*).

Figure 7C shows the averaged percent alpha (startle) responses recorded from the conditioned and pseudoconditioned mice. Alpha responses are activity within 50 ms of CS onset that were at least 4SD above the mean baseline activity. Over the training sessions, conditioned and pseudoconditioned mice typically exhibited alpha responses on less than 25% of the trials, with no significant differences between conditioned and pseudoconditioned mice (groups, $F_{(1,15)}$=2.502, *ns*). The repeated measures ANOVA did, however, reveal a significant interaction of groups and sessions due to the decrease in pseudoconditioned responses and the maintenance of alpha responses at about 25% for conditioned mice (groups*sessions, $F_{(1,9)}$=2.074, p<.05). The increase in alpha responses during session 10 is likely due to the short onset latency of the well-developed CR.

Figure 7D shows the averaged percent short latency response recorded from both groups of mice. Short latency responses reflected activity between 50-70 ms following CS onset that was 4SD above the mean baseline activity. A repeated measures ANOVA revealed that conditioned animals exhibited more short latency responses than pseudoconditioned animals over the ten training sessions (groups, $F_{(1,15)}$=5.377, p<.05; session, $F_{(1,9)}$=3.920, p<.0005; groups*session, $F_{(1,9)}$=3.158, p<.005). This may reflect an earlier onset time of the CR, as the conditioned group exhibited larger CRs with each training session.

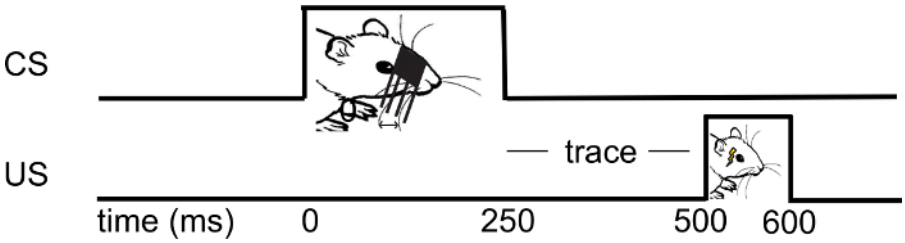
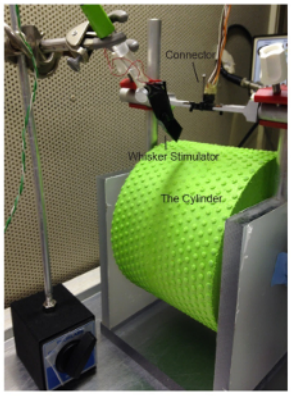


Figure 1: The Trace Eyeblink Conditioning Paradigm. In trace eyeblink conditioning, an innocuous conditioned stimulus (CS) is paired with a mildly noxious unconditioned stimulus (US) meant to induce an eyeblink response. The CS and US are separated by a stimulus-free temporal gap, making the paradigm hippocampus-dependent[23]. In the paradigm described here, a 250 msec long whisker vibration CS is paired with a 100 msec long periorbital shock US. The CS and US are separated by a 250 msec long temporal gap. Please click here to view a larger version of this figure.

A



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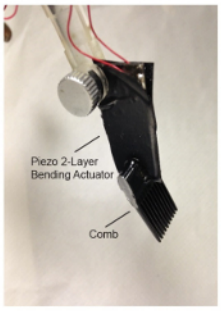

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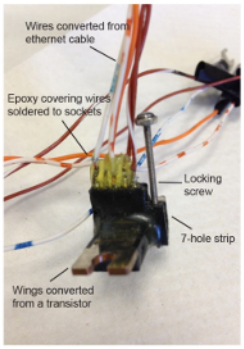

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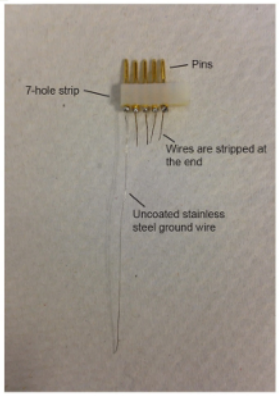
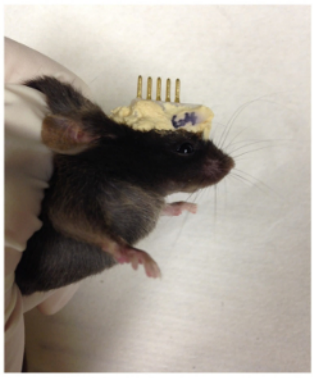


E

7th Hole	6th Hole	5th Hole	4th Hole	3rd Hole	2nd Hole	1st Hole
Ground	Shock	Shock	EMG	EMG	Blank	Locking Screw

F



G

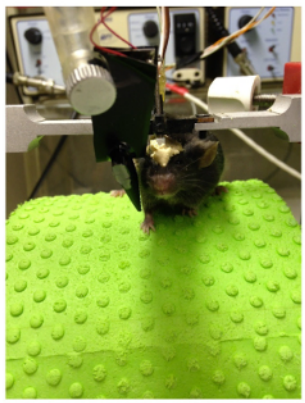


Figure 2: The Head-fixed Preparation. A, The head-fixed set-up for eyeblink conditioning with the cylinder, the whisker stimulator, and the connector indicated. **B**, The whisker stimulator with the two-layer bending actuator and comb indicated. **C**, The connector with the individual parts indicated: locking screw, the 7-hole strip, the wires soldered to the sockets and epoxy to preserve the wires, the wings converted from a TO-220 style transistor. **D**, The headpiece with the individual parts indicated: The 7-hole strip, the pins, and the wires soldered into the pins, including the uncoated stainless steel ground wire, and the individual shock and EMG stainless steel wires, with 0.2 cm of polyimide coating stripped off the end. **E**, The configuration for each hole in the 7-hole strip for both the connector and the headpiece. **F**, A mouse with a surgically implanted headpiece. **G**, A mouse is placed on the cylinder, ready to be trained. Please click here to view a larger version of this figure.

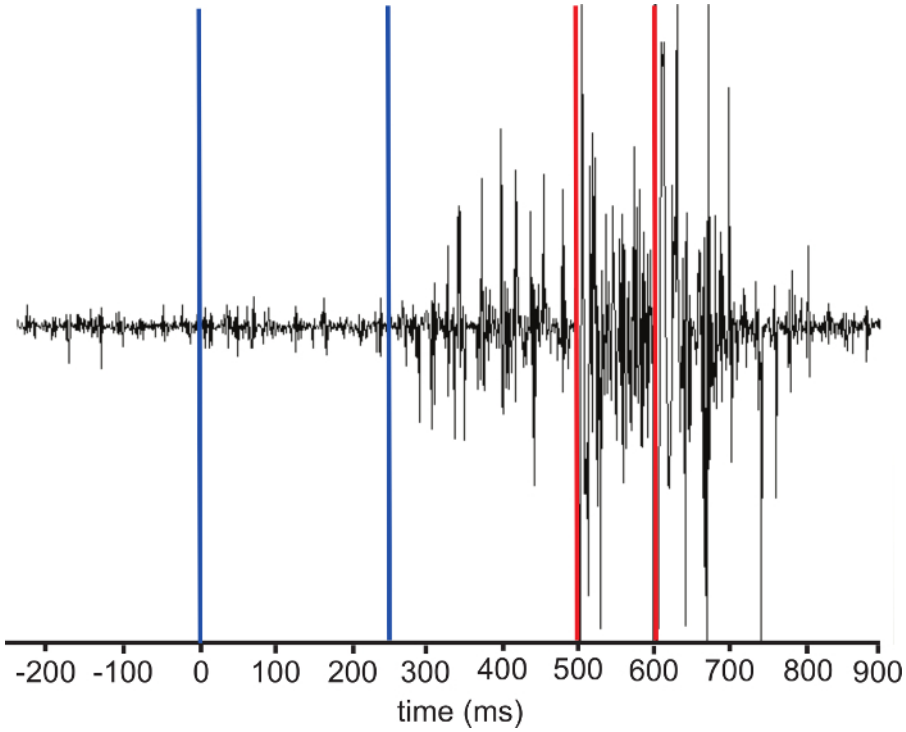


Figure 3: Example EMG recording. The blue lines delineate the CS presentation (250 msec long). The red lines delineate the US presentation, which shows the artifact that comes from the electrical shock (100 msec). The CS and US are separated by a 250-msec stimulus-free interval. Within this stimulus-free interval is higher amplitude EMG activity (relative to baseline) which delineates a CR. Please click here to view a larger version of this figure.

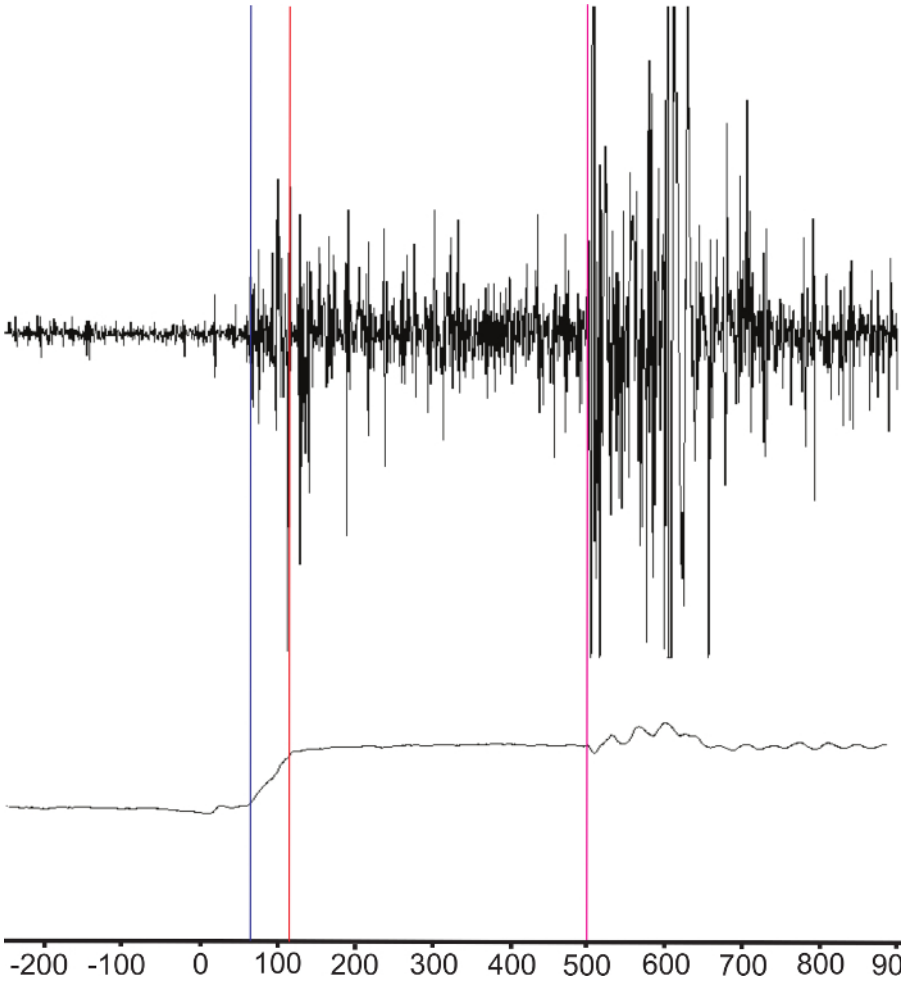


Figure 4: Example EMG and corresponding optical infrared reflection recordings. The EMG recording is just as accurate as the optical infrared reflection sensor in detecting conditioned response onset (blue line), latency to response peak (red line), and unconditioned eyeblink response onset (pink line). Please click here to view a larger version of this figure.

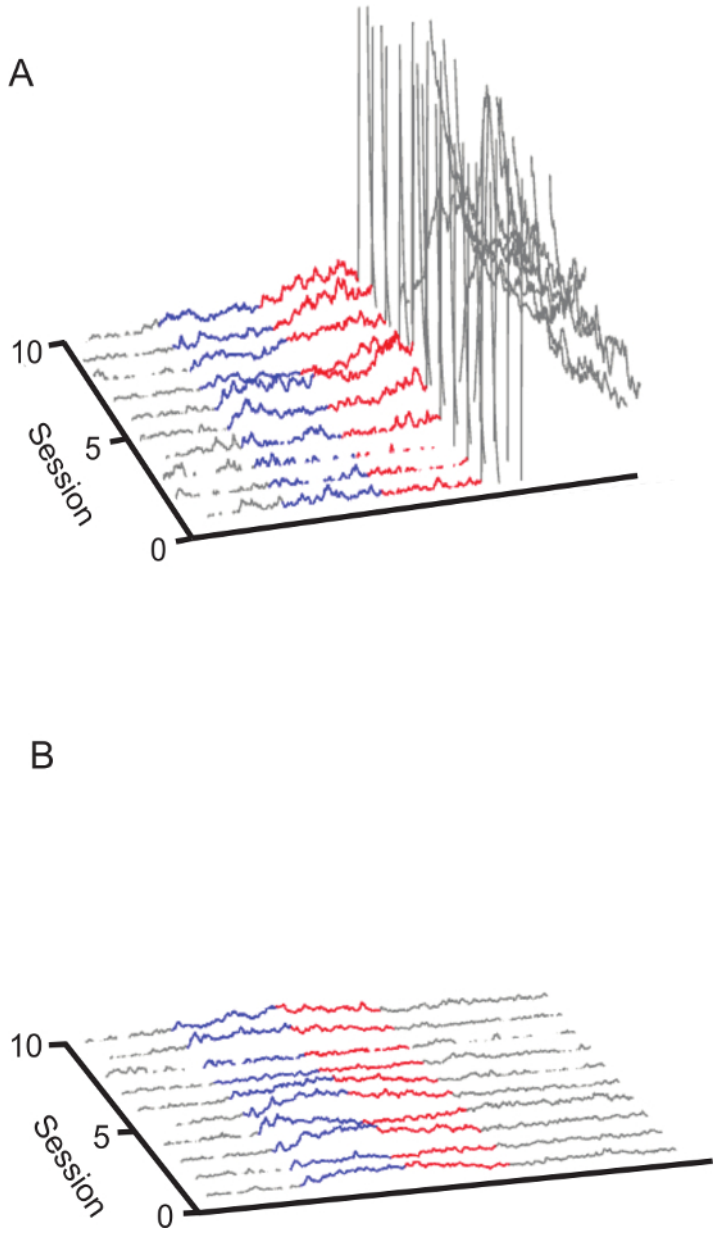


Figure 5: Averaged Eyeblink Traces. Averaged rectified and integrated traces of the eyeblink response for one conditioned mouse (**A**) and one pseudoconditioned mouse (**B**). Each trace represents the average response of the mouse for all trials over a single session. The blue section represents the response during the whisker vibration CS presentation. The red section represents the response during the stimulus-free trace interval. In **A**, the shock US induced an artifact that is present during the dark grey area after the trace interval. **B** shows the responses to the whisker vibration CS only. Presentation of CS alone and US alone trials was pseudorandomized in pseudoconditioning. Please click here to view a larger version of this figure.

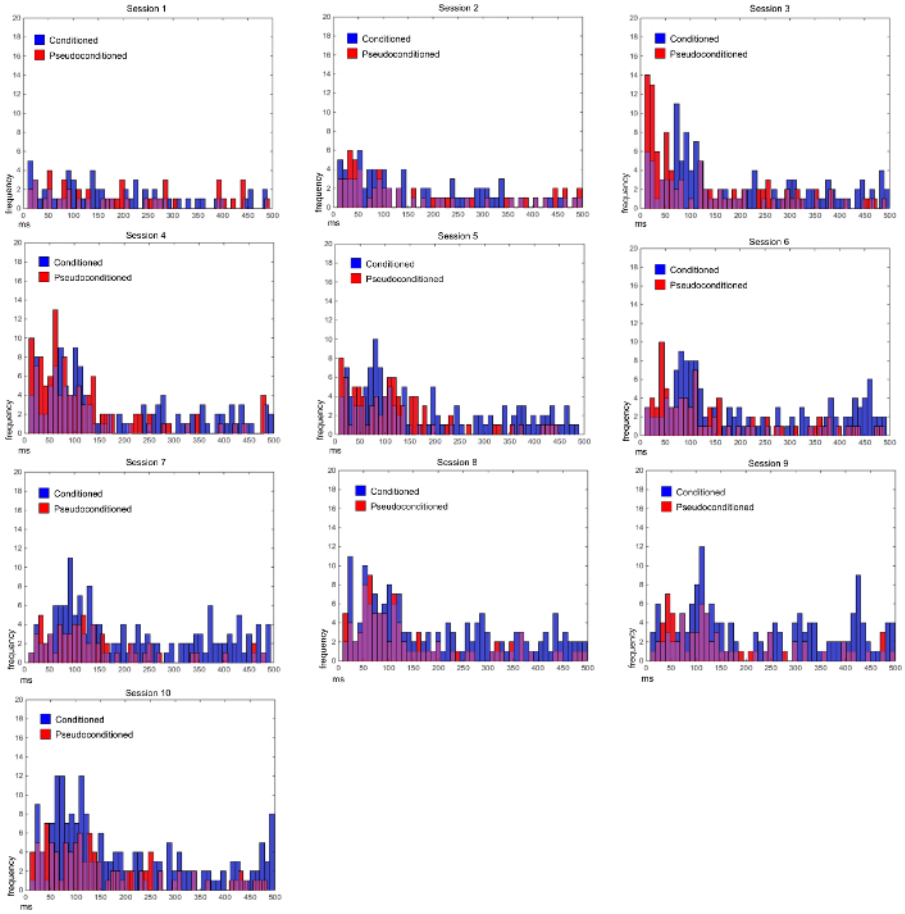


Figure 6: Histograms of the Response Peak Times. Histograms of the time to the response peak for all conditioned (n=8; blue bars) and 8 pseudoconditioned (red bars) animals for all trials across all sessions (data from one pseudoconditioned mouse was excluded to allow a direct comparison of the number of responses between groups). Purple bars indicate where the conditioned and pseudoconditioned animals overlap. Response peak times are calculated as the time of the largest peak of the rectified and integrated EMG recording between CS and US onset. Please click here to view a larger version of this figure.

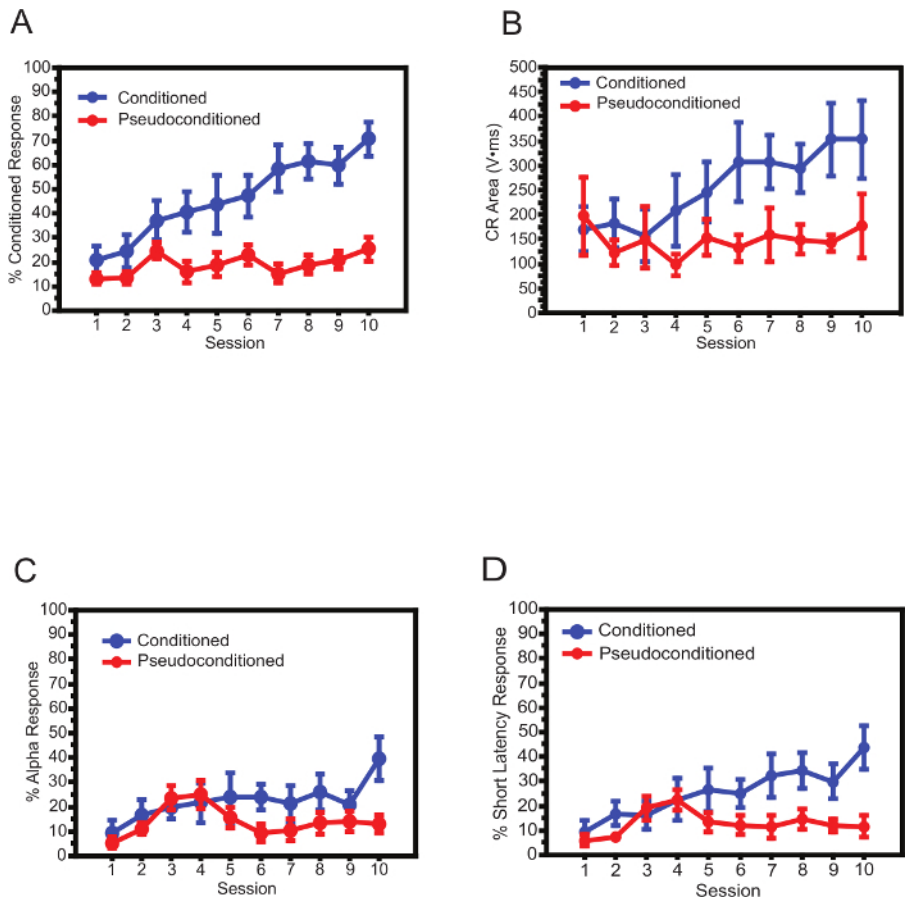


Figure 7: Response Curves of Conditioned Animals (n=8; blue curves) and pseudoconditioned animals (n=9; red curves). A, Percent Averaged Adaptive Conditioned Responses (± SEM) for conditioned and pseudoconditioned animals over 10 training sessions. **B**, Averaged area (± SEM) of the CR for conditioned and pseudoconditioned animals over 10 training sessions. **C**, Percent Averaged Alpha Response (± SEM) for conditioned and pseudoconditioned animals over 10 training sessions. **D**, Percent Averaged Short Latency Responses (± SEM) for conditioned and pseudoconditioned over 10 sessions. Please click here to view a larger version of this figure.

Discussion

Classical eyeblink conditioning is a form of associative learning that is a useful tool for understanding the neural substrates underlying learning and memory. Previous methods employed for eyeblink conditioning in rodents such as mice involved a chamber that allowed for the animal to move about freely. A head-fixed preparation for eyeblink conditioning in mice, using the apparatus described by Chettih *et al.* and Heiney *et al.* and most recently utilized in light-evoked trace eyeblink conditioning in Siegel *et al.* offers several advantages, such as allowing for certain procedures and experiments to be performed during eyeblink conditioning that had not been possible or had been limited in the past (*e.g., in vivo* two-photon calcium imaging)[14-16]. This type of preparation also facilitates the use of other techniques such as *in vivo* electrophysiology with tetrode arrays. As mice appear to become distressed from restraint, this head-fixed preparation was developed to keep heads secure while reducing the amount of restraint required. While this preparation allows mice to ambulate on a cylindrical treadmill, others have also successfully collected behavioral data from head-fixed mice on a stable platform[24].

There are, however, certain issues that arise with the use of the head-fixed cylinder system. One concern is that when one initially places an untrained mouse on the cylinder, it may need some time to learn to rest, walk, or run on the novel apparatus. Most mice learn quickly to rest, walk, or run on the cylinder comfortably. Two habituation sessions help to accustom the mice to the cylinder and by the middle of the second session, most mice are comfortable on the cylinder. Another concern that arises is headpiece avulsion. There has been some problem with this in the past during multi day training sessions, but the use of the adhesive luting cement has ameliorated issues with headpiece avulsion. The use of the adhesive luting cement has resulted in 0% headpiece avulsions from more than 50 surgeries since using this technique. With correct use and sufficient spread of the cement on the cranium, researchers should encounter no issue with headpiece avulsion. Finally, by presenting the CS during habituation sessions, there may be some concern about latent inhibition. It is clear, however, that the mice were conditioned successfully (see **Figure 7**). Note that the intensity of the whisker vibration can be modified by changing the amount of voltage being passed by the rheostat. If the mice are unable to learn, the voltage can be increased to support successful conditioning while also minimizing startle responses. Researchers can also change the type of electrical shock US that is delivered to the animal. The electrical shock US can be a single electrical pulse lasting 100 msec as reported here. Alternatively, as the stimulus isolator utilized in this protocol is capable of transmitting biphasic pulses, researchers can also employ 120 Hz 6 biphasic pulse pairs at 1 msec per pulse for an effective electrical shock US as well.

Boele *et al.* indicated the likelihood that certain "conditioned responses" may arise from startle (alpha) responses to the CS or short latency responses (SLR)[25]. Alpha responses are considered to be rapid eyelid closures within a 50 msec latency after CS onset. Mice often keep their

eyes closed for the duration of the trial, following this alpha response. SLRs, on the other hand, have a latency of 50-70 msec from CS onset and typically arise after only a few CS-US pairings. The short latency to onset of both alpha and SLRs and the fact that SLRs can arise after only a few CS-US pairings indicate that they are likely not driven by the hippocampal-cerebellar circuitry. These observations reviewed by Boele *et al.* therefore beg the question of whether the CRs recorded here are learned responses. As **Figure 7C** and **7D** show, neither alpha responses nor SLRs account for a majority of the CRs recorded, suggesting that the adaptive CRs are mediated by the forebrain and cerebellum. The histograms in **Figure 6** which show latency to the peak of the eyeblink response following CS onset also indicates that while certain trials may include an early onset alpha or short latency response, with more training trials, the peak of the eyeblink response actually occurs later and closer to the onset of the US, indicating the development of well-timed conditioned responses. As mentioned previously, whisker stimulation and shock intensity were attenuated to a level that was not startling or overly aversive, but remained effective in producing adaptive conditioned responses. Finally, as **Figure 7B** shows, the size of the CR (measured as the area under the curve in the rectified and integrated response, as in **Figure 5**) is larger in the conditioned group than in the pseudoconditioned group once the conditioned group reached a learning criterion of 60%, signifying that these responses require several sessions to learn, unlike SLRs and alpha responses.

Boele *et al.* also pointed out that EMG measurements, while an accurate method of blink detection in larger animals such as rabbits, are not as practical in small rodents such as mice[25]. They recommend the use of the magnetic distance measurement technique (MDMT) to avoid the detection of false positive signals that EMG recording may indicate. The MDMT technique, while impressive in its sensitivity and quality of blink detection, also presents the disadvantage of having to anesthetize the animal each day of training in order to attach the chip[26]. This in itself may confound learning rates. We find that the EMG signals recorded with the procedures described here are easily obtained, of high temporal resolution, reliable and relatively easy to measure and analyze.

Figure 4 shows a representative EMG signal detecting blinks with a corresponding signal from an infrared reflective optical sensor placed close to the mouse's eye during training. There is a clear correlation in blink detection between the EMG signal and the optical sensor, denoting the accuracy of blink detection using EMG recordings. An advantage of EMG recordings is that it allows for the greatest temporal resolution. Although the resolution is degraded by integrating over a 10 msec time constant, one can also analyze the raw EMG data to detect eye blink activity. Spike count is one parameter that may be used to detect CRs[18]. One disadvantage with EMG recordings is that signals will undoubtedly be contaminated by the electrical artifact coming from the electrical shock US (see **Figures 3 - 5**). This, however, does not impede the ability to record conditioned blink responses, *i.e.,* those that occur prior to onset of the US. Another disadvantage in employing EMG recordings is that, using the present criterion for CR detection, a noisy baseline may hide what may otherwise have been detected as a CR.

The head-fixed preparation described here is similar to that presented by Heiney *et al.*[15] There are, however, certain notable differences from their elegant system. For example, the method described here for recording eyeblink responses are wires placed subdermally above the eye to record EMG activity. This technique allows for reliable and stable recordings of eyelid activity and thus, of the eyeblink response. Proper placement and fixation of these wires ensures quality recordings that last for at least two weeks, the duration of the experiment. An advantage of using EMG wires rather than a high-speed camera as used by Heiney *et al.* is that EMG recordings have extremely high temporal resolution, and do not require the daily positioning and calibration that a camera requires[15]. The camera does, however, offer direct visual determination of eyelid closure.

Another difference between the two systems is the method for CS delivery. This particular head-fixed mouse preparation utilizes whisker stimulation as the conditioned stimulus. The results demonstrate that mice can be conditioned effectively with whisker stimulation as the CS, just as head-fixed rabbits can be conditioned with whisker vibration[27]. Heiney *et al.* demonstrated stimulation of the whisker pad as an effective CS by directing a weak puff of air to the whisker pad[15]. Although both techniques demonstrate effective conditioning, placing a comb over selected whiskers and vibrating the comb allows for the ability to stimulate individual whisker rows or even individual whiskers. This technique has been used in previous studies to allow for animals to serve as their own control (*i.e.,* the stimulated whisker row was compared to a row of unstimulated whiskers)[20].

In summary, a head-fixed preparation for whisker-signaled eyeblink conditioning allows for a secured cranial platform for performing advanced techniques and experimentation that had previously been impossible or difficult to perform. Mild whisker stimulation was used as the conditioned stimulus and a mild electrical shock was used as the unconditioned stimulus. Eyeblink responses were recorded with wires placed subdermally behind the eyelid. Reliable learning was demonstrated in mice with direct stimulation of selected whiskers as the CS and periorbital electrical shock as the US, and no learning was evident in mice that were given random presentations of the CS and the US. The EMG recordings provided a dependable and relatively simple method for recording eyeblink responses and observing how the responses changed across training sessions.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was funded by the Department of Defense (W81XWH-13-01-0243) and the National Institutes of Health (R37 AG008796). We thank Alan Baker in Northwestern University's machine shop for building the head-fixed cylinder apparatus. We thank Dr. Shoai Hattori for his guidance in MATLAB and Solidworks. We thank Dr. John Power for the LabView software that controlled the experiment.

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