Video Article

The Use of Trace Eyeblink Classical Conditioning to Assess Hippocampal Dysfunction in a Rat Model of Fetal Alcohol Spectrum Disorders

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Abstract

Neonatal rats were administered a relatively high concentration of ethyl alcohol (11.9% v/v) during postnatal days 4-9, a time when the fetal brain undergoes rapid organizational change and is similar to accelerated brain changes that occur during the third trimester in humans. This model of fetal alcohol spectrum disorders (FASDs) produces severe brain damage, mimicking the amount and pattern of binge-drinking that occurs in some pregnant alcoholic mothers. We describe the use of trace eyeblink classical conditioning (ECC), a higher-order variant of associative learning, to assess long-term hippocampal dysfunction that is typically seen in alcohol-exposed adult offspring. At 90 days of age, rodents were surgically prepared with recording and stimulating electrodes, which measured electromyographic (EMG) blink activity from the left eyelid muscle and delivered mild shock posterior to the left eye, respectively. After a 5 day recovery period, they underwent 6 sessions of trace ECC to determine associative learning differences between alcohol-exposed and control rats. Trace ECC is one of many possible ECC procedures that can be easily modified using the same equipment and software, so that different neural systems can be assessed. ECC procedures in general, can be used as diagnostic tools for detecting neural pathology in different brain systems and different conditions that insult the brain.

Video Link

The video component of this article can be found at https://www.jove.com/video/55350/

Introduction

It is quite hard to imagine that in today's day and age with better health care and access to health services, alcohol abuse remains a major global health concern. Unfortunately, it has been shown that an expectant mother who drinks a high amount of alcohol can have a child with severe brain damage and neurodevelopmental disorders that last a lifetime, as evident in those afflicted with fetal alcohol syndrome (FAS)[1,2,3]. In women with some confirmed history of maternal alcohol use, the developing fetus is also susceptible to small amounts of alcohol or different patterns of alcohol consumption that produce varying differences in blood alcohol concentrations. In this latter case, while the children may not exhibit the severe morphological or neurobehavioral disruptions as those with FAS, they may still exhibit lifelong cognitive disabilities and emotional disturbances that range from mild to severe[3,4]. Altogether, FAS and less severe forms of prenatal alcohol-mediated disruptions constitute a collection of fetal alcohol spectrum disorders (FASDs). It is no surprise that FASDs are completely preventable, but astonishingly estimates show that in populations where alcohol abuse is quite common, they remain the primary non-genetic cause of neural and cognitive disability, affecting about 2% to 5% of young US children and those in European countries such as France and Sweden. With respect to the incidence of FAS alone within the US, the prevalence is 2 to 7 per 1,000 live births[5], implying that the overall incidence of FASDs to be much higher than that for FAS.

Neuroimaging studies conducted in children with FASDs have shown that they display brain abnormalities, such as a thinner corpus callosum[6], smaller anterior cerebellar vermis[7], and smaller hippocampus[8]. These brain abnormalities underlie some of the long-term neurocognitive disruptions observed in children with FASDs. The exact links that tie variations in maternal alcohol-mediated brain changes and variations in the profile (i.e., type, extent) of particular neurocognitive impairments have yet to be clearly determined. But as a starting point, the hippocampus is an excellent candidate for determining its susceptibility to prenatal alcohol effects. Indeed, children with FASDs exhibit deficits in hippocampal-mediated behaviors such as place learning[9,10] and delayed object recall[11].

Rodent models of FASDs have proven to be invaluable in elucidating the mechanisms leading to neurocognitive disruptions seen in children with FASDs. A well-established binge-exposure model that we have adopted involves delivering alcohol to rats during postnatal days 4-9[12,13], a period when the brain undergoes rapid synapse and dendritic contact formation, comparable to human fetal week 24 and extending into the 3rd trimester[14,15,16,17]. This particular model induces significant loss of hippocampal neurons[18,19] and neurons in many other brain regions such as the cerebellum[12,13,14,15,16,17,18,19,20,21,22,23], accompanied by severe impairments in cognitive functions spanning different domains[21,24,25]. Cognitive disruption from early alcohol exposure in rats may be assessed in different ways, particularly with *eyeblink classical conditioning* (ECC). ECC is a paradigm that has been utilized for more than a century to scientifically investigate the fundamental basis of learning[26,27] and as such, provides a useful method to better understanding the adverse neurocognitive consequences resulting from fetal alcohol exposure. It is a very flexible paradigm that allows investigators to use a variety of different ECC procedures, any of which can be

examined across many mammalian species ascending the phylogenetic scale (from mice to humans) and over different courses of brain development[28,29,30,31]. Furthermore, the fundamental neural circuits that mediate associative learning in this paradigm are supported by experimental and neuropsychological reports in these same species[26,32,33,34,35,36,37].

One form of ECC, trace ECC is demonstrated in this paper (**Figure 1**). To provide context, it is compared against the more traditional form - delay ECC. The ECC paradigm was modeled after classical conditioning using dogs, first carried out by the Nobel-Prize winning physiologist, Ivan Pavlov. Pavlov discovered that certain stimuli such as tones do not naturally elicit salivation, but when it precedes and overlaps with the delivery of food, the salivary response can be strengthened from repeated presentations of the two, provided that this tone-food contingency is maintained. This is an example of delay ECC, with the notion that associative strength is mediated by immediate temporal contiguity between the two stimuli, thus making learning conditions optimal for an animal. He also tested other variations of the tone-food contingency, such as turning the tone off and leaving a "trace" period before delivering the food. When these two stimuli were discontiguous enough, it became much harder for the dogs to emit salivation responses prior to the delivery of the food. The discontiguity between the tone being turned off and the delivery of the food is thus an example of trace ECC. As rodents do not naturally salivate to the presence of food, more species-relevant stimuli such as mild shock are used instead; they also do not naturally emit defensive eyeblink responses to tones. With this backdrop, rodent ECC procedures involve presenting a tone at a given decibel level and pairing it in some fashion with mild shock to either the eyelid muscle (*orbicularis oculi*) or the *temporalis* muscle to elicit an eyeblink response. The tone is considered a *conditioned stimulus* (CS) while the shock is considered an *unconditioned stimulus* (US). In delay ECC, the CS is presented first; this stimulus remains on for a given duration. Afterwards, the US is delivered. These two stimuli overlap for a given duration, and then both terminate simultaneously; the resultant eyeblink response emitted due to the US is considered an *unconditioned response* (UR). In this procedure, rodents learn to emit eyeblink responses sometime after the CS is presented, but just before the US, in order to anticipate this aversive stimulus. The learned eyeblink response is referred to as a *conditioned response* (CR). For trace ECC, the CS and US are separated by a period of time that is void of stimuli known as a *trace* interval; they do not overlap in time as in delay ECC. During this interval, the animal is tasked to resolve the associational requirements between stimuli. Similar to delay ECC, learning occurs when the animal consistently emits a blink response after the CS turns off, but immediately before delivery of the US. Over some amount of acquisition training (CS paired with US), learning curves (i.e., based on different CR measurements) develop. Lesion and neuroimaging studies show that successful learning in delay ECC is dependent on having intact cerebellar-brain stem neuro-circuitry[38,39,40], whereas trace ECC is a higher-order procedure that requires additional neural engagement from the hippocampus[41,42,43,44] and other cortical structures[45,46]. Because of the timing-related requirements needed in order to acquire trace CRs successfully, this task is also more difficult to learn (even for normal subjects).
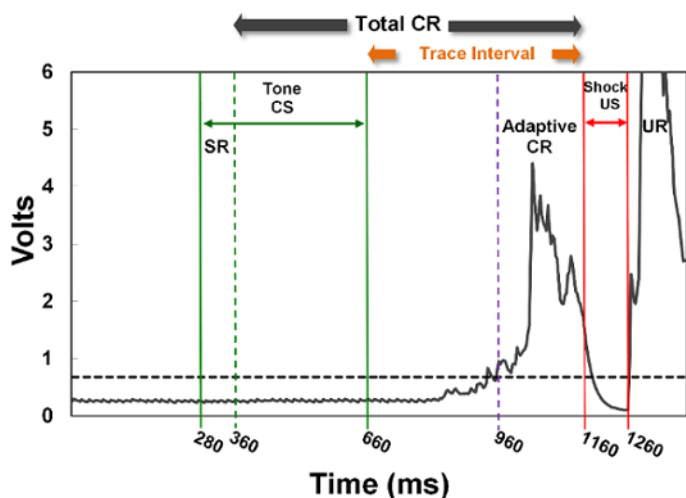
Figure 1: Trace eyeblink classical conditioning. An actual waveform is shown that is representative of an adult rat in the unintubated-control (UC) group. The tone CS (85 dB, 2.8 kHz) is first presented for 380 ms. A trace interval of 500 ms ensues, where no stimuli are present. Afterwards a shock US (1.6 mA) is delivered for 100 ms. Successful learning in this task occurs when the frequency (%) or amplitude (in volts) of eyeblinks during the conditioned response (CR) time window (Total CR period) increases over many sessions of training. In particular, rodents with an intact hippocampus will usually emit more well-timed CRs (Adaptive CRs) just prior to the onset of the shock US (within a 200-ms window). Startle responses (SRs) during the first 80 ms after tone CS onset and unconditioned responses (URs) are also measured. Non-associative SRs are typically low or nonexistent in well-trained rodents, while URs are expected to be high in frequency and amplitude. This task requires that the rodent learn to bridge the association between the CS offset and US onset (during the trace interval), therefore making it inherently more difficult to acquire compared to delay ECC. Please click here to view a larger version of this figure.

Here we demonstrate the adverse functional consequences of neonatal alcohol exposure that is delivered in a binge-like manner, as assessed by a trace ECC procedure that delivers an 85 dB tone CS (2.8 kHz) which remains on for 380 ms, followed by a 1.6 mA shock US which remains on for 100 ms, and these stimuli are separated by a trace period of 500 ms. We have reported on the utility of this behavioral assay in previous studies examining choline intervention and iron supplementation in mitigating the effects of neonatal alcohol exposure[18,47]. Indeed, trace ECC can be used as a diagnostic tool to assess neonatal alcohol-induced hippocampal pathology. The advantage it has over delay ECC is that it is more sensitive to detecting disturbances in hippocampal function, which is compromised in humans with FASDs.

Demonstration of ECC extends far outside the fetal alcohol field. Many variants of ECC (*e.g.*, delay, trace, compound, reversal) can be used to elucidate ontogenetic differences in learning across development, the neurobiological basis of associative learning in normal mammals, as well

as the vulnerabilities of different brain systems to many challenges, including (but not limited to) teratogens, environmental toxins, traumatic brain injury, neurodegenerative diseases, and psychiatric conditions.

Protocol

NOTE: All procedures were approved and carried out in accordance with the policies set forth by the East Carolina University IACUC. Long-Evans rats were generated from females mated with male breeders. Pups from all three treatment groups (see 1.1) were generated within a litter from the same dam. Five litters were produced and each litter was culled to 8 pups on postnatal day (PD) 3. The remaining two pups from each litter were assigned to separate experiments. Both male and female offspring (one per exposure group) were included in the study. A total of N = 27 adult offspring were examined in this study; 3 rats were excluded due to broken 3T wire leads (see 3.1.1) which were irreparable on Day 1 of ECC training.

1. Preparation of Groups, Materials, and Solutions

1. On PD 3, remove pups from the dam and place them on a thermo-regulated water heating pad to keep warm. Use a random selection procedure to place rat pups into one of three groups: (1) alcohol-intubated (AI); (2) sham-intubated control (SI); or (3) unintubated-control (UC). Carry out approved procedures for identification, such as [non-toxic] ink tattooing of their paws with a 30G x 1/2 in needle following a numbering scheme. Return the pups to the dam when finished with tattooing.
2. **Prepare an intragastric intubation tube(s) using polyethylene (PE) size 50 and 10. The overall length of this tube is user-dependent and can be adjusted accordingly.**
   1. Use micro dissecting scissors to cut a 3 in piece of PE-50 tubing and a 6 in piece of PE-10 tubing.
   2. Carefully attach the PE-50 tubing to a sterile 22 G x 1 in hypodermic needle.
   3. Insert one end of the PE-10 tubing into the open end of the PE-50 tubing. A diagonal cut may be needed at the tip of the PE-10 tubing in order to direct it into the PE-50 tubing.
   4. Cut a small piece of PE-50 tubing (about 2-3 mm) and insert it on the open end of the PE-10 tube. This will serve as a visual stopper.
   5. Store the complete intragastric intubation tube in fresh 70% isopropyl alcohol to keep it sterile.
3. **Carry out aseptic procedures to prepare a stock milk solution in 50 mL bottles. The stock milk solution is based on a diet formula first established by West et al. (1984)[48]. Store the bottles at -18 °C until time of use. Thaw two bottles of milk using warm water prior to use.**
   1. Draw out 7.16 mL of 95% ethyl alcohol (USP) using a sterile syringe and add it to one 50 mL milk bottle. This makes an 11.9% v/v alcohol solution that is delivered to rat pups over the first two feedings of the day (see 2.1.1); the total daily dose of alcohol is 5.25 g/kg/day. It is designed to deliver the alcohol in a volume (0.0278 mL/g per feeding) in enriched milk solution.
      NOTE: This amount can be adjusted based on litter size and expected usage over 3-4 days. The amount of alcohol added must also be adjusted in order to make an 11.9% v/v solution.
   2. Shake this bottle well and label it accordingly (*i.e.*, 11.9% v/v Ethyl Alcohol in Milk).
   3. Label the second bottle of milk (without alcohol added) accordingly (*e.g.*, Milk-Only) and use it for supplemental feedings as indicated in 2.1.1.
   4. Store both milk bottles in the refrigerator.

2. Neonatal Alcohol Exposure (Postnatal Days 4-9)

1. **Give AI and SI pups 4 total intubations per day starting on PD 4. Separate each intubation by 2 h. Because AI pups are intubated with an actual solution, each of their intubations are considered feedings.**
   1. Intubate the AI pups with the 11.9% v/v Ethyl Alcohol in Milk solution during the first two feedings of the day, and then intubate them with the Milk-Only solution during the last two feedings of the day to supplement their growth.
   2. Intubate the SI pups 4 times without any solution every two hours, ensuring the same duration of PE-10 tube exposure that AI pups endure. The UC group does not receive any intubations.
2. **Remove pups from the dam and place them on a thermo-regulated water heating pad to keep warm. Weigh each pup and record its body weight (in g). Refer to a pre-made feeding chart to determine the intubation volume for each AI pup and note it in a record sheet.**
   1. Place the 11.9% v/v Ethyl Alcohol in Milk solution in a warm water bath and shake it well.
   2. Intragastric intubation procedure:
      1. Flush out the intragastric intubation tube that was stored in 70% isopropyl alcohol well with warm water.
      2. Extract the correct amount of solution using a sterile 1 mL syringe.
      3. Dip the tip of the intubation tube (PE-10 end) in fresh corn oil (this facilitates insertion).
      4. Measure the length of the PE-10 tube from the pup's mouth to its stomach. Adjust the PE-50 stopper to guide with the stopping point.
      5. Carefully insert the PE-10 tube into the pup's mouth, proceeding down its esophagus, slightly passing through the gastroesophageal sphincter, and into its stomach. Examine the pup, stabilize it, and depress the syringe plunger to deliver the solution. Do this at a slow rate.
         NOTE: To avoid accidentally inserting the tube into the trachea, first direct the tube so that it curves and makes contact with the soft palate prior to proceeding forward. Use the palate as a guide, as the tube will naturally deflect off this region and be guided down the esophagus. Otherwise, any substantial deviation of the tip ventrally may cause it to enter into the trachea. It is best to remove the tube and not to proceed with the intubation if one is not certain about its entry position - reinsert the tube correctly on the next attempt.

6. Carefully remove the PE-10 tubing and examine the pup for backwash of solution, blood, or physical injury. Replace pup with its littermates if it is fine. The entire intubation procedure takes approximately less than 1 min for each pup and approximately 4 min total for the 4 intubation-treated pups in each litter (2 AI, 2 SI).

7. Intragastric intubation procedure for SI pups: Follow the same PE-10 insertion procedure as that for AI pups, but without any solution for the same duration (1 min).

8. Return all pups back to the dam immediately after completing procedures for the last pup in the litter. Return the dam to the vivarium until the next feeding session (in 2 h).

9. Flush out the intragastric intubation tube with warm sterile water and replace it in 70% isopropyl alcohol for storage. Flush the tube well with water prior to each feeding session.

10. Repeat 2.2 (except weighing pups) to 2.2.2.9 for the second feeding session. At the last two feedings, intubate the AI pups with the Milk-Only solution using the same intubation volumes determined in 2.2.

3. Weigh the rats at regular intervals such as on PD 15 (when eyes open), PD 21 (weaning), PD 30, PD 60, and PD 90 (surgery) to obtain representative growth curves.

3. Fabrication and Modification of Electrodes

1. **Fabricate one electromyographic (EMG) "headstage" for each rat (Figure 2). The headstage allows for recording of eyeblink responses via the eyeblink system (Figure 4).**
   1. Construct a headstage that consists of two size 3T polytetrafluoroethylene (PTFE)-coated stainless steel wires (5 cm each), one size 10T PTFE-coated stainless steel wire (5 cm), three male contact pins, and one micro strip socket insulator that is cut-down to 3 holes (see 3.1.4).
   2. Strip off the PTFE coating from the 10T wire by grasping the center with a serrated high precision tweezer and removing the coating in both directions with the smooth high precision tweezer. Ensure that all traces of coating have been removed from this wire.
      1. Crimp one end of the 10T wire to a contact pin with a serrated platform dental plier; this will serve as a ground wire. Hold a 3T wire carefully with the smooth high precision tweezer 1 to 1.5 mm from one end. Strip off the 1 to 1.5 mm PTFE coating while leaving the rest of the coating intact. Crimp a contact pin to the exposed end of the 3T wire.
      2. Carry out the same procedures for the second 3T wire. Both wires will serve as the positive and negative leads of the EMG recording electrodes.
   3. Perform tug tests on all wires to ensure that they are secured to the pins. Take caution not to bend or damage the wires.
   4. Cut a segment of a socket insulator strip down to 3.5 holes (cut the middle of the fourth hole) with the cutting blade of a wire stripper. Scale down this segment to just 3 full holes and sand-down both sides if necessary. This helps to ensure that 3 full holes are available. Insert pin-wire units into the three holes of the micro strip until the crimped ends are flushed with the bottom edge of the insulator segment. The 10T assembly needs to be in one outer hole of the micro strip and not in the middle.

2. **Modify bipolar stimulating electrodes (Figure 2).**
   1. Give each rat one bipolar stimulating electrode. This electrode applies a small amount of electrical current via a stimulus isolator (see eyeblink system) as the shock US during ECC. The electrodes are acquired from a manufacturer in twisted form and are shielded.
   2. Untwist the two metal leads 2-3 times to make a V-shape (spread 5 mm) and then use the smooth high precision tweezer to straighten each "prong" as much as possible. Scrape off 1 mm of shielding from each prong's tip (all around) with a razor blade. Inspect each prong for irregularities and bends, and correct if necessary without scraping off additional shielding.

3. Remove manufacturing oil and foreign debris from the EMG headstages and bipolar electrodes by washing them in 95% ethyl alcohol. Allow them to dry then autoclave.
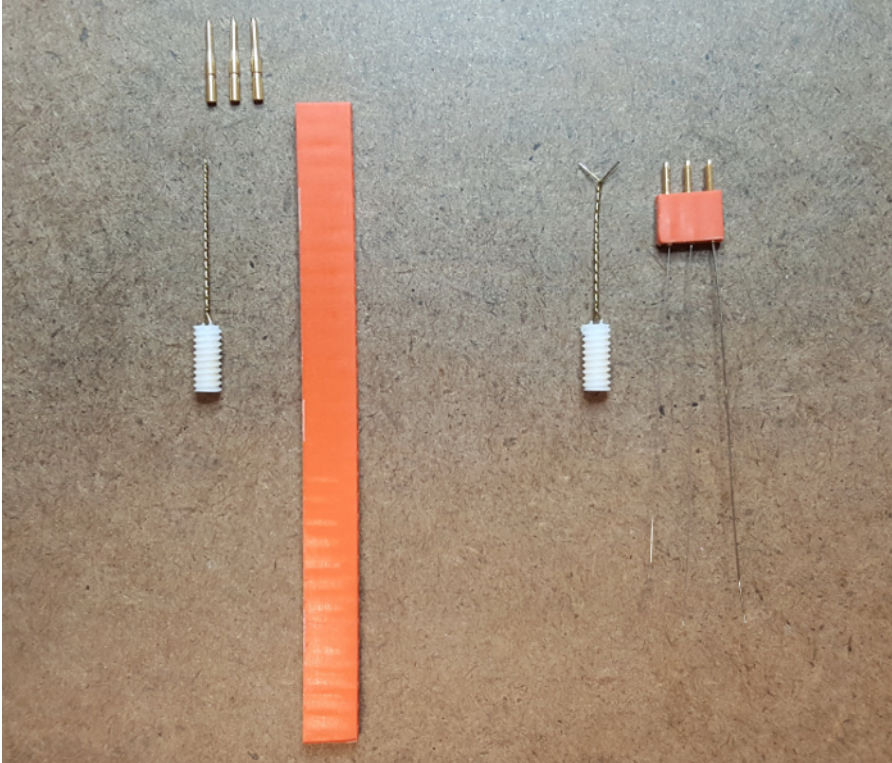
Figure 2: Electromyographic (EMG) recording electrodes and bipolar electrode. The finished EMG headstage (right, orange) is constructed from three male contact pins, two size 3T PTFE-coated wires, one size 10T PTFE-coated wire, and a modified micro strip. The three wires are approximately 5 cm each and are crimped to the contact pins. The finished bipolar electrode (right, white) is untwisted, re-straightened, and molded in a V-shape (5 mm split). Shielding is removed from the tips of the two prongs. Please click here to view a larger version of this figure.

4. Eyelid Surgery Procedure (Postnatal Day 90)

1. **Preparation of materials (see Materials for detailed supply information), animals, surgical, and non-surgical areas:**
   1. Autoclave surgical instruments and supplies - the number in parentheses ( ) indicates approximately how much is needed per adult rat or measurement: surgical instruments (1 set per 4 rats, as approved by the ECU IACUC), gauze pads (3-4), cotton-tipped swabs (5-6), 20 x 20 wrap (4 per/surgery session), porcelain crucible (1), stainless steel microspatula (1), 0.9% saline (1 wash bottle), Petri dish (1), and 0-80 stainless steel screws (3/rat).
   2. Other sterile supplies needed: surgical blade size 10 (1 per 4 rats), surgical drape (1 per rat), veterinary ophthalmic ointment, povidone-iodine (1 wash bottle), 26G x 3/8" hypodermic needle (2), nickel-plated pin vise with #55 drill bit, nickel-plated flathead jeweler's screwdriver (1.8-2 mm blade).
   3. Prepare a sterile holding area for all surgical tools and supplies using an approved research/medical grade disinfectant. Place materials on this space when the area is ready.
   4. Prepare a sterile surgical space using an approved research/medical grade disinfectant. This space contains a thermo-regulated water heating pad, stereotaxic apparatus fitted with a rat anesthesia mask, glass bead sterilizer, and isoflurane gas vaporizer (for anesthesia). The vaporizer has tubes to the anesthesia mask and to an induction chamber located on a separate space for non-sterile animal preparation procedures; each tube is controlled by its own valve.
   5. Scrub hands and arms thoroughly with antimicrobial soap. Without touching the inside, pre-open any items that have been packaged in sterilization pouches or wraps. Don sterile gloves using aseptic procedure and transfer all pertinent items to the surgical area. Set the drill bit at the proper depth in the pin vise (~ 2 mm) and prepare the screwdriver. Cover the sterile materials with a sterile wrap until the surgery is ready to begin.
   6. Prepare a non-sterile space with a weighing scale, electric fur trimmer, sterile 1 mL syringes with 26G x 3/8 in needles (1 per rat), surgery log, and buprenorphine (0.03 mg/mL concentration) administered 0.1 mL/100 grams.
      CAUTION: Buprenorphine is a DEA schedule III semisynthetic opioid, and must be locked and logged appropriately.
   7. Check the isoflurane vaporizer for appropriate gas level; add more gas if it is low. Leave the gas flow and mixture knobs off for now. Turn on the O_2 tank and check that there will be enough gas for all surgeries.
   8. Weigh each rat and record its body weight in the surgery log. Use a buprenorphine injection chart to determine the proper injection volume.
   9. Turn on the vaporizer valve to the induction chamber and leave the valve to the surgery mask off. Turn the mixture to 3 and flow rate to 3 (3% isoflurane with 100% O_2 as the carrier gas at a volume of 3 L/min).
   10. Place a rat in the induction chamber and allow it to reach proper anesthetic plane (slow breathing, lack of pedal reflex, lack of blink reflex). Remove the rat from the chamber once it has reached anesthetic plane.
   11. Shave its head using the fur trimmer, exposing a sufficient amount of skin for the incision site and the left eyelid. If necessary, place it back in the induction chamber to reach anesthetic plane again before resuming with the trimming.

12. Disinfect the exposed skin by applying alternating rubs of isopropyl alcohol and povidone-iodine three times. If necessary, place it back in the induction chamber to reach anesthetic plane again, prior to transferring to the surgery table.

2. **Surgery**
    1. Turn on the vaporizer valve to the anesthesia mask and shut off the valve to the induction chamber. Adjust the mixture to 2.5 and flow rate to 2 (2.5% isoflurane with 100% O_2 as the carrier gas at a volume of 2 L/min). Transfer the rat to the stereotaxic apparatus and follow standard procedures in securing its head to the incisor bar and ear bars (see Geiger et al., 2008[49] for an example).
    2. Give the rat a pre-surgical injection of buprenorphine (SC) and carefully dispose of the needle (uncapped) in a sharps container. Place a sterile surgical drape on the rat, exposing just the surgical area and isolating it from the rest of the body.
    3. Don a surgical mask, surgical cap, surgical gown, goggles, and any other personal protective equipment required by the IACUC. Wash and scrub hands and arms thoroughly with antimicrobial soap. Don sterile gloves using sterile procedure.
    4. Apply a small amount of ophthalmic ointment to both eyes using a cotton-tipped swab to prevent them from drying.
    5. Proceed with the surgery when the rat exhibits proper anesthetic plane. Monitor the rat continuously throughout the surgery and check the isoflurane level periodically. Adjust the flow and mixture knobs accordingly during surgery based on the rat's response to the anesthesia.
    6. Make an anterior-posterior incision at the midline of the cranium with a scalpel blade. This incision should expose enough area in front of the eyes (i.e., exposing the frontal bone) and slightly behind the lambdoid suture.
    7. Scrape away the periosteum on top of the cranium carefully not to cause excessive bleeding. Wipe off excess connective tissue and blood with a cotton-tipped swab.
    8. Drill a hole using the drill bit with the aid of a pin vise, starting directly behind the coronal suture on one parietal bone. Remove any blood and bone debris with a cotton-tipped swab. Grasp a 0-80 screw by the threading with splinter forceps and fasten it to the hole with the jeweler's screwdriver; tighten-down the screw just enough without damaging cortical brain tissue (usually 3-4 full turns).
    9. Follow the same procedures described in 4.2.8 to fasten two more 0-80 screws to the skull, one directly behind the coronal suture of the opposite parietal bone and one directly anterior to the right lambdoid suture. It has been found that 3 screws are sufficient for anchoring the dental cement (see 4.2.18) to the cranium, as a fourth screw (anterior to the left lambdoid suture) would impede the placement of the bipolar electrode.
    10. Remove an EMG headstage from the Petri dish. Face the headstage so that the 10T wire is on the rat's right side. Bend the 10T wire upward, making it parallel with the bottom edge of the micro strip. Give it a slight bend to the right side so that the wire can come around the anterior and posterior 0-80 screws. Set it aside but close enough to reach.
    11. [Assuming one is right-handed] Place 3 in dressing forceps in the left hand and 4 in dressing forceps in the right hand. Grasp the upper skin at the incision site of its left eye with the 4 in dressing forceps and direct the 3 in dressing forceps towards the corner of this eye; grasp the skin at this corner. Maintain the grasp with the 3 in dressing forceps while releasing the 4 in dressing forceps.
    12. Take one 26G x 3/8 in needle and insert it through the corner of the eyelid; rotate the needle so that the beveled side is face-up. Use the micro dissecting forceps with platform to insert the middle 3T wire into the needle's hole. Push it through the hole a few centimeters without going down completely; this wire is the negative lead.
    13. Carry out the procedures described in 4.2.11 and 4.2.12 to insert the outside 3T wire into the middle portion of the eyelid; this wire is the positive lead.
    14. Grasp both needles with one hand while grasping the headstage with the other hand (or forceps). Pull the needles away from the eyelid while guiding the headstage in the same direction in one continuous movement. If necessary, rotate the headstage so that the 10T wire is towards the animal's right eye; do not allow the 3T wires to cross. Use fine tipped forceps to double-check that the positive lead is in the middle of the eyelid.
    15. Adjust the headstage so that it is centered between the eyes and is in an optimal position atop the frontal bone, anterior to the bilaterally-inserted screws. Hold the headstage with one hand and hold the 3 in dressing forceps with the other hand. Wrap the 10T wire around one or both screws on the 10T side.
    16. Place 3 in dressing forceps in one hand and 4 in dressing forceps in the other hand. Use the 3 in dressing forceps to grasp the skin at the incision site towards the left temple. Use the 4 in dressing forceps to create a small "pocket" by separating the skin (i.e., superficial fascia) from the temporalis muscle. Use the iris scissors to cut away more connective tissue, working in towards the corner of the left eye, to increase the size of this pocket. Be careful not to go in too deep or cut any blood vessels.
    17. Take a bipolar electrode and shape the wire leads so that they can be fitted along the curvature of the temporalis muscle, while the two prongs are situated (dorsal and ventral) posterior to the left eye (see video), and the bottom end of the bipolar electrode can be situated straight atop the cranium. Allow enough distance between the headstage and this electrode for the commutator cable plugs (**Figure 3**) to attach without impediment.
    18. Pour dental cement powder into the crucible - start with about 1/3 of the crucible, and then scale the amount as necessary. Add the liquid component until the consistency is watery, and mix these components with the spatula. When the cement's consistency becomes more viscous, apply it to the incision site - covering all edges of the skin with at least 1/8 in borders.
        1. Apply enough cement to fortify the headstage and bipolar electrode without preventing the rat from closing both of its eyes. Quickly wipe off any cement that drips onto unwanted areas of the rat's fur.
    19. Ensure that at least 4-5 threads of the bipolar electrode are left unsealed by cement so that the bipolar plug can be twisted on (**Figure 3**). Remove excess cement from the headstage - the top of the micro strip and the three contact pins must be clean. The cement hardens quickly and may easily prevent full coupling of any of these electrodes to the commutator cable plugs. Use splinter forceps to remove excess cement that has partly dried.
    20. Wait for the cement to harden; it becomes clear. Use the micro dissecting scissors to snip off excess length from the two 3T wires, leaving a few centimeters of working room.
    21. Grab the micro dissecting forceps with platform on the left hand while grabbing the micro dissecting forceps with fine points on the right hand. Grasp one 3T wire with the left forceps slightly in front of the eyelid. With the right forceps, push the eyelid up to expose more 3T wire and hold it there. Release the left forceps and use the left hand to grasp the headstage (ensure it is secure by now). Use the right forceps to strip off some PTFE shielding, which allows the wire to make contact with the *orbicularis oculi* muscle.
        NOTE: Take caution not to pull too hard on a 3T wire, as it may detach from the contact pin. If a wire detaches, then it will not be possible to replace the contact pin as the hardened cement will not release freely from the cranium without causing bone breakage.

22. Perform the steps in 4.2.21 to strip off excess PTFE from the second 3T wire.
23. Grab the two micro dissecting forceps as stated in 4.2.21 (with platform on left, fine points on right). Position the left forceps slightly in front of one 3T wire while leaving room for the right forceps to be positioned directly in front of the eyelid. Grasp the wire with both forceps.
    1. Create a "hook" by keeping the right forceps still and rotating the left forceps up and around towards the eyelid; do this in one motion. Carefully release the right forceps, and then the left forceps.
    NOTE: Hooking the electrodes on the eyelids help to minimize the possibility of them rubbing onto the cornea. This also minimizes the possibility for wire breakage during any point in the behavioral testing phase. While hooking does not fully prevent the wire from rubbing against the cornea for all animals, they typically show no signs of eye damage as reflected by their high blink amplitudes (measured by the digital oscilloscope in 5.1.7) and from daily post-surgical health observations (excessive tearing, partial eyelid closure, lack of mobility from eye damage). If a rat shows initial signs of discomfort, it is anesthetized with isoflurane (see 4.1.10) and its electrodes are re-examined and/or re-positioned. Ophthalmic ointment is applied to prevent the cornea from drying. The rat is observed daily for any post-procedural eye complications and should they persist, then seek veterinary care. Rats that exhibit severe eye complications which cannot be corrected with electrode re-positioning or with veterinary treatment, are humanely euthanized according to the standard operating procedures of the ECU IACUC.

24. Perform the steps in 4.2.23 for the second 3T wire. Snip off excess wire using the micro dissecting scissors.
25. Use one hand to release the rat from the ear bars while supporting its body with the other hand. Inspect its head for any blood or debris, clean up if necessary, and give it a post-operative injection of buprenorphine.
26. Place the rat in a recovery bin that has a source of heat (such as a thermo-regulated water heating pad) to aid recovery. Mark in the surgery end time in the surgery log and any pertinent notes about the rat during surgery. Monitor the rat for proper respiration and when it exhibits sternal recumbency or moves about, it may be returned to its home cage.
27. Re-sterilize the instruments (except micro dissecting forceps - as they may be damaged) using the bead sterilizer and ensure that the surgical area remains aseptic, and repeat aseptic procedures if multiple eyelid surgeries will be performed.

5. Trace Eyeblink Classical Conditioning Procedure
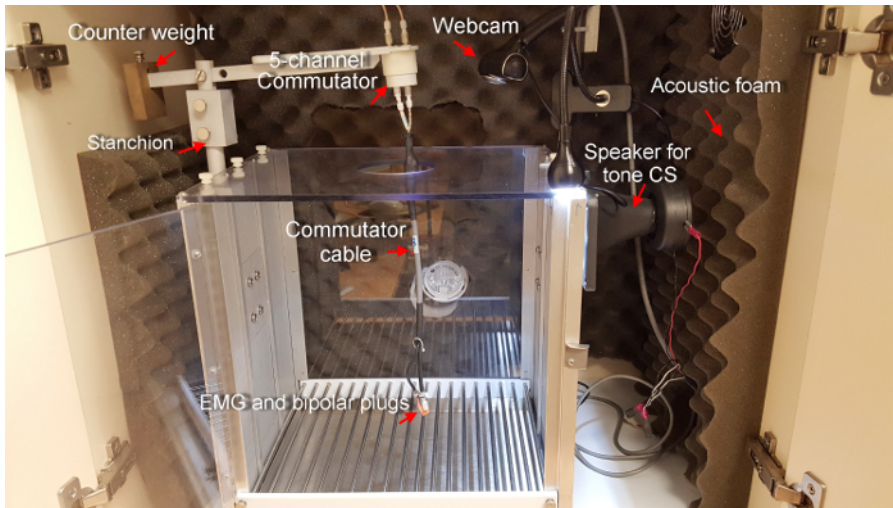
Figure 3: Modified operant conditioning box for eyeblink conditioning. Rats are freely-moving mammals, and therefore a rotating commutator is used for maintaining electrical signal contact from the EMG and bipolar plugs that are attached to the head. The commutator is attached to the arm of the stanchion, which is counter-weighted for alleviating pressure on the rat. A piezo tweeter (speaker) delivers a 2.8 kHz tone at 85 dB and these values are calibrated regularly. Acoustical foam assists with attenuating environmental noise. Please click here to view a larger version of this figure.
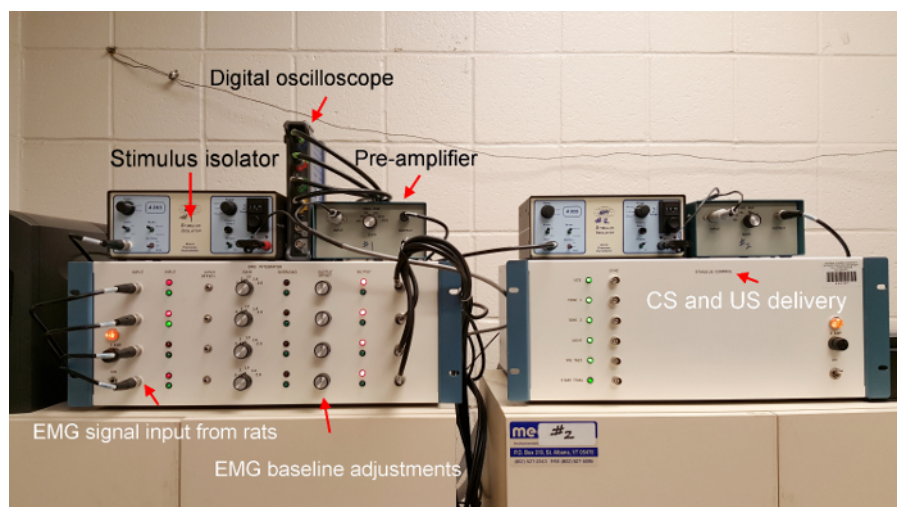
Figure 4: Eyeblink conditioning system. This custom-built system consists of an EMG Integrator unit that filters and amplifies incoming signals from the rats, a Stimulus Control unit that delivers various stimuli in addition to tones and shocks, a pre-amplifier for each operant box to increase EMG signal gain, and a stimulus isolator for each operant box; it provides varying shock levels (in mA). A digital oscilloscope (not part of the stock eyeblink system) is used for diagnostic purposes during habituation and acquisition. Please click here to view a larger version of this figure.

1. **Begin habituation and acquisition training 5 days after surgery. Give each rat one day of handling and habituation to the training apparatus - consisting of a modified operant conditioning box that is housed in a larger sound-attenuated chamber (Figure 3) - and follow with 6 days of acquisition training. Carry out the habituation and acquisition sessions using a custom-built eyeblink system (Figure 4).**
    1. Create a training log file and print it out to use for daily record keeping. This log should have the squad of rats pre-assigned pseudo-randomly (or other randomization method) to their training boxes.
    2. Power on the eyeblink system, computer, and run the eyeblink software. This version of the software has different modules (or applications) that run independently. Run the Animal module to create a *.RAT file that contains subject information (session number, animal ID, sex, weight, and box number). A single RAT file manages up to four rats, which comprises a squad of subjects. Enter the values accordingly and save the file. Create a separate RAT file for each squad of rats to be trained.
    3. Transfer the rats from their vivarium to the testing room and close the door. Handle each rat for 5 min.
    4. Transfer a rat to its designated box and connect the EMG and bipolar plugs from the commutator cable to the corresponding EMG headstage and bipolar electrode on its head.
       NOTE: On first exposure to the commutator cable, a rat may struggle and show signs of stress. Handle the rat carefully and allow breaks in between unsuccessful attachments to alleviate its stress.
    5. Use a commutator that rotates 360° while maintaining electrical signal delivery/reception as a rat moves freely within a box. It has 5 channels (3 for the EMG electrodes, 2 for the bipolar electrodes) leading into the EMG Integrator unit, which filters and amplifies the raw signal.
    6. Determine that the rat is moving freely, and that it is not hindered by the weight of the commutator cable assembly or stanchion arm. Adjust the counter weight at the back of the stanchion if necessary. Check that all plugs are secure then close the box door. Perform the same connection/check procedure for all rats.
    7. Use an oscilloscope to observe their EMG activity for abnormal signals (e.g., high frequency and/or high amplitude electrical activity) and a video surveillance system to check their status (e.g., motor activity, sensitivity to environment, signs of pain) if available. Note any problems that are observed in the training log.
    8. Allow rats to habituate to their chambers for 10 min, and then return them to their homecages.
    9. Clean and disinfect the operant boxes, sweep the floor, and clean the table(s). Carry out these procedures after every session.

2. **Acquisition training occurs over 6 consecutive days (sessions).**
    1. Day 1: Transfer a rat to its designated box and connect the EMG and bipolar plugs from the commutator cable to the corresponding EMG headstage and bipolar electrode on its head. Carry out the same procedures indicated in 5.1.6 and 5.1.7.
    2. Turn on the stimulus isolators and set the current to be delivered at 0.4 mA (4% of 10 mA as shown in video). The isolators deliver electrical current in alternating fashion (i.e., from trial to trial), between the dorsal and ventral prongs of the bipolar electrode. This helps reduce muscle fiber fatigue (at each muscle site) over repeated stimulus deliveries.
    3. Observe all eyeblink responses emitted and tolerances exhibited on each trial for all rats. After every second trial, increase the shock US intensity by 0.4 mA for each rat, until 1.6 mA is reached (on Trials 7 and 8). Double-check that all rats are responding normally in their boxes, that all amplification and gain settings are constant, and for proper electrical signals (i.e., low/little EMG noise) coming from them. Use a shock US intensity of 1.6 mA for all days of acquisition training.
       NOTE: If a rat exhibits pain to a given shock level (e.g., jumping high off the floor, climbing on walls, running around), the trial is paused and it will be returned to the previous mA setting (0.4 mA below) for two trials. Afterwards, the rat will receive the higher shock value that it did not tolerate again and if tolerance is shown, it will receive increasing values until 1.6 mA is reached. If it cannot tolerate any value, then the next lower value (in 0.4 increments) at which it tolerates will be used throughout training.
    4. Use an oscilloscope to observe their EMG activity for abnormal signals (*e.g.*, high frequency and/or high amplitude electrical activity) and a video surveillance system to check their status (*e.g.*, motor activity, sensitivity to environment, signs of pain).
       NOTE: Explanation of Trace ECC: A representative trial epoch with captured EMG waveforms is illustrated in **Figure 1** and the trace ECC procedure is explained in detail in the video. The trace conditioning training file is set to deliver 100 trials (90 paired CS-US trials

and 10 CS-only trials on every 10th trial) with an average inter-trial interval of 30 sec. The entire training session lasts approximately 52 min (assuming no stoppages). All rats are naïve in Session 1 and will develop differential learning curves (as determined by the expression of CRs) as they progress through several days of training.

5. Start the training: Run the Blink software module. When prompted, select the correct RAT file and the training file for "trace" eyeblink conditioning.
6. Write down the start time and the experimenter's name in the training log. Monitor all activity at this point: proper EMG signals, good blink responses to the shock US (i.e., high/frequent URs), health status of the animals, and proper hardware function. Write any noteworthy remarks in the training log.
7. Data collection is captured automatically by the software as RAW files (one per rat, per day). The RAW files contain EMG amplitude (in volts), frequency counts, latency, and areal data for startle responses (SRs), URs, and CRs (total and adaptive). Shock artifact obscures the first 100 ms of the UR period, therefore only the last 140 ms of the UR period contain UR data.
8. At the end of training, the software will automatically close. Remove all rats from their training boxes and return them to the vivarium.
9. Repeat steps 5.2.5 - 5.2.6 for all subsequent training days. Before starting the Blink module on a given day, update the session number within the RAT file. This allows for creation of new RAW files for all rats that day. Each rat should have 6 RAW files, assuming that none has been removed from the training.

3. **Process the RAW files and create a file that has been filtered for statistical analysis.**
    1. Run the Analysis module. Review each session for each rat on a trial-by-trial basis for problematic trials. This is carried out to screen for trials that may be compromised due to various reasons (*e.g.*, excessive pre-CS baseline activity, excessive movement, electrode detachment, cabling/wire issues). The software has algorithms that assist the experimenter with detecting and removing "bad trials" from being part of the filtered data set.
    2. Import the filtered data into a spreadsheet program and perform pertinent statistical analyses.

Representative Results

The eyeblink software is capable of providing a large and comprehensive set of data for many types of measurements. For brevity, we report in this study, representative results for learning and performance measures that include adaptive CR percentage, adaptive CR amplitude, UR percentage and UR amplitude. The adaptive CR period was chosen as it represents the acquisition of well-timed eyeblink responses over repeated training, as a result of enhanced synaptic plasticity in the hippocampus during trace ECC[50,51,52]. The UR measures were chosen to elucidate whether the neonatal alcohol-induced learning deficits in trace ECC were due to disruptions in associative learning or disruptions in responding to the shock US - which may indicate motivational or motor differences, rather than learning differences among treatment groups. Data for each measure were analyzed using 2 (Sex) x 3 (Neonatal Group) x 6 (Session) mixed ANOVAs, with Session as the repeated-measures factor. Significant main effects for neonatal treatment were analyzed using Tukey's post hoc tests and significant interactions were analyzed using simple effects tests. All statistical analyses were conducted using a minimum alpha level of 0.05 and results in graphs are mean ± SEM.

Beginning with the adaptive CR percentage measure, the ANOVA indicated a significant main effect of neonatal group, $F_{(2,21)} = 11.69$, p <0.001, but no significant main effect of sex ($p = 0.71$) or significant interaction between these factors ($p = 0.20$). As expected, adaptive CR percentage increased over the six sessions of training, $F_{(5, 105)} = 81.15$, p <0.001, and the differences among neonatal groups were dependent on some level of session, $F_{(10, 105)} = 4.58$, p <0.001. There were no other significant interactions involving the session factor. Likewise for adaptive CR amplitude, there was again a significant main effect of neonatal group, $F_{(2,21)} = 22.32$, p <0.001, but no significant main effect of sex ($p = 0.21$) or significant interaction between these factors ($p = 0.48$). CR amplitude also increased significantly over the six sessions of training, $F_{(5, 105)} = 59.27$, p <0.001, and the differences among neonatal groups were dependent on some level of session, $F_{(10, 105)} = 4.31$, p <0.001. Overall, both CR measures showed significant differences among the group means and these means separated significantly at different sessions of training. To confirm which groups differed significantly, Tukey's post hoc tests showed that the alcohol-intubated (AI) rats performed significantly worse on both CR measures than the unintubated-control (UC) and sham-intubated (SI) rats (p <0.01 for CR percentage; p <0.001 for CR amplitude), which did not differ from each other (p's >0.05). Simple effects tests performed on the significant Neonatal Group x Session interactions for both CR measures, confirmed that the AI rats were more significantly impaired in acquiring CRs beginning at Session 2 and carrying on through Session 6 compared to both UC and SI rats (all p's <0.05), which did not differ from each other throughout six sessions. The only exception was adaptive CR amplitude for SI rats did not begin to differ significantly from AI rats until Session 3. These results are shown in **Figure 5A**, **5B**.

There were no significant differences in the UR measures due to sex, neonatal group, or interactions of these factors with the session factor. These negative findings indicated that each group was able to emit eyeblink responses to the shock US equally, and that the learning deficits observed in the AI rats were not influenced by motivational or motor differences in blinking (**Figure 6A**, **6B**).
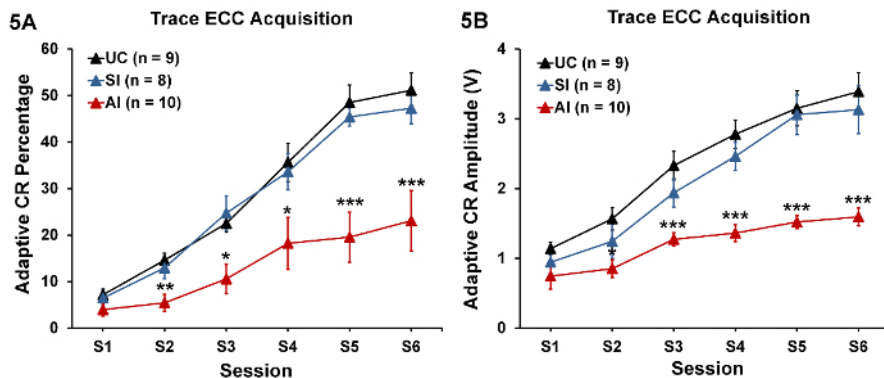
Figure 5: Acquisition of trace conditioned responding (mean ± SEM). Early alcohol exposure (group AI) significantly affected acquisition of adaptive conditioned response (CR) percentage (A) and amplitude (B). Trace ECC is inherently difficult to acquire, therefore the measures are relatively lower for all groups - with delay ECC, percentages may reach 80-85% in rodent models of FASD[21,53]. Nonetheless, the trace ECC procedure is more taxing on the hippocampus, which is susceptible to alcohol effects during early brain development. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ between UC and AI rats; sample sizes are provided in parentheses. Please click here to view a larger version of this figure.
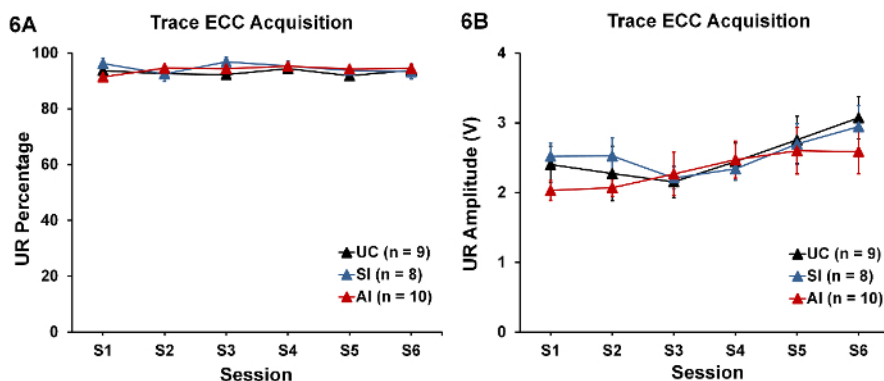
Figure 6: Acquisition of unconditioned responses (mean ± SEM). Eyeblink performance (UR percentage and UR amplitude) was not significantly different among groups. The lack of differences indicate that the shock intensity used during acquisition training did not differentially alter motivation in the AI rats or their ability to produce defensive blink responses to the shock, compared to both control groups (UC and SI). Sample sizes are provided in parentheses. Please click here to view a larger version of this figure.

Discussion

Neonatal rat pups that received ethyl alcohol during postnatal days 4-9 exhibited trace eyeblink conditioning impairments in adulthood. These findings support the idea that alcohol is a teratogen with enduring detrimental effects on hippocampal function. Overall, conditioned responding in the trace procedure was lower for rats exposed to alcohol compared to rats in both control groups. The associative learning impairments in the alcohol-exposed rats were not influenced by motivational or motor differences (*i.e.*, no differences in blinking to shock US intensity).

While trace ECC is a useful diagnostic tool for elucidating challenge-induced hippocampal neuropathology, the results from this method must be placed into proper context. First, the key procedural elements in this demonstration involved the targeted delivery of alcohol during a known window of vulnerability to the developing brain, fabrication of electrode hardware that allows recording of electromyographic activity and delivers shock, surgical implantation of the aforementioned hardware, and subsequent animal testing using a learning paradigm that assesses a cognitive function of interest. At each stage in the process, care must be taken to not cause unnecessary/unintended harm to the rodent subjects and to monitor their health signs regularly. Their behavioral results provide the "window" to cognition, a psychological construct that is only accurately described when their health is not compromised by experimental errors encompassing alcohol dosing, hardware defects, or surgical implantation. Thus, each procedural element in the research process must be implemented in a sound manner in order to ensure that results from ECC can be extrapolated to findings in humans. Secondly, the ECC paradigm provides insight on the nature of associative learning, but care must be taken not to extend findings using this approach and broadly ascribe them to other cognitive domains - such as working memory, short/long-term memories, and consciousness - unless one has incorporated some facet of these domains within an ECC study by experimental design. For example, this demonstration examined the acquisition phase of trace ECC learning, but did not examine memory retention in the rats after they completed the training. Memory is thus an independent psychological process that should be evaluated in addition to learning. By design, one may incorporate a memory retention interval to assess either short-term or long-term memory ability. Thirdly, recognition that there are parallel memory systems[54] that may work simultaneously along with motivational, experiential, and hormonal factors which contribute to behavior, is essential for understanding that associativity (during ECC) is but one of many processes that reveal what is "good" or "poor" about learning. Lastly, trace ECC is not a purely hippocampal-dependent task, as other brain regions may mediate some component of the CR. Thus, an understanding of the interactions between different neural circuits and/or the type of stimulus parameters that are utilized in a study, must be taken into account when making implications based on discrete results. The cerebellum, for example, also contributes to trace ECC, where it influences the topographical characteristics of the CR and CR timing, particularly when the ISI is short in duration. Trace ECC is not affected in

humans with cerebellar damage who are tested with a long trace interval (1,000 ms), but is affected in those who receive a shorter trace interval (400 ms)[34]. Furthermore, bilateral lesions of the dorsal medial prefrontal cortex (mPFC) that target the anterior cingulate and medial agranular regions in mice, prevent acquisition of trace CRs[55], while destruction of the caudal mPFC in rabbits produces similar results[46]. These findings also highlight the importance of considering species differences in prefrontal contributions to cerebellar-brain stem driven associative learning, such as trace ECC. While neonatal alcohol exposure during PD 4-9 adversely affected acquisition of 500-ms trace CRs for adult rats in this study and others[47,56], this is not the same case for neonatal alcohol-exposed rats that experience a 300-ms trace interval, even when challenged at a relatively high dose of alcohol (5 g/kg)[57], suggesting that the trace impairment in alcohol-exposed rats is dependent on the duration of the trace interval.

In this study, the hippocampus was emphasized as being vitally important for mediating trace ECC, and when challenged by neonatal alcohol exposure, exhibits neural-related damage as reflected by impairments in acquisition of trace CRs. It must cautioned, however, that the cerebellum-brain stem circuitry, particularly the interpositus nucleus, is essential for many facets of ECC, including the acquisition, expression, and topographical features of the CR, depending on the type of ECC task including trace ECC[36,40,55,58,59]. Indeed, this neural circuit interacts with the hippocampus for driving the expression of CRs during higher-order forms of ECC, such as trace ECC[60]. Whether alcohol exposure during early brain development specifically affects hippocampal function in trace ECC is not entirely clear. Many different brain regions are vulnerable to early alcohol insult, including the mPFC, cerebellum, and hippocampus[18,19,23,47,61,62], and it is very likely that alcohol disrupts the functioning of these structures to varying degrees and to varying, but functionally important differences across many ECC procedures. In spite of the pitfalls regarding the interpretation of results from trace ECC studies, successful acquisition of trace CRs has been shown to at least rely on an intact hippocampus, as supported by animal lesion studies[42,44,63,64,65]. This procedure thus remains a highly valuable approach for demonstrating the links between developmental alcohol exposure to trace conditioned responding because the neural circuitry underlying it, is much better understood than that of other hippocampal-dependent tasks, such as place learning in the Morris water maze, novel object recognition, and contextual and trace fear conditioning.

ECC as a behavioral method to "assay" cognition, has widespread applicability in the field of developmental neuroteratology. Indeed, recent findings from our lab support the notion that the developing hippocampus is highly sensitive to alcohol effects, which may be mitigated by different interventional strategies[18,47]. The key benefit here is that with a better understanding of alcohol-induced trace ECC learning deficits, they may be predictive of other problems in hippocampal-based functions outside of associative learning - particularly those known to be mediated by the same hippocampal neurocircuitry.

Application of trace ECC and its other variants (e.g., delay, reversal, discrimination, compound) to elucidate the neurobiological mechanisms and neural systems involved in associative learning, can be extended beyond the field of fetal alcohol research. For example, this paradigm has received much attention in human cases and animal models of psychiatric conditions such as schizophrenia[66,67], neurodegenerative diseases such as Alzheimer's disease[68,69], and drugs of abuse[70,71,72]. Its benefits as a research method to assess neurocognitive function and dysfunction are thus evident across many psychological and biomedical disciplines, including neuroscience.

Disclosures

The authors have nothing to disclose.

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