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Research report

Effects of neonatal alcohol dose and exposure window on long delay and trace eyeblink conditioning in juvenile rats

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HIGHLIGHTS

- Both trace and long-delay eyeblink conditioning are impaired in a rat model of FASD.
- First study of different doses and developmental periods of alcohol exposure.
- Findings relate to brain injury and eyeblink conditioning deficits in human FASD.

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ABSTRACT

Classical eyeblink conditioning has been used to assess learning and memory impairments in both humans and animal model studies of fetal alcohol spectrum disorders (FASD). Gestational exposure to alcohol in humans and its equivalent in rats severely impairs various eyeblink conditioning tasks, but less is known about how these effects are influenced by variables, such as the timing and dose of alcohol exposure. In a series of four experiments, we systematically examine how varying the timing and dose of alcohol exposure impact long delay and trace eyeblink conditioning in juvenile rats, tasks that both depend on a brainstem-cerebellar circuit but differ in that trace conditioning additionally recruits the hippocampus and prefrontal cortex. Using a "third-trimester-equivalent" alcohol exposure model, rats were exposed to a high binge dose of alcohol at one of two alcohol doses over postnatal days (PD) 4–9 or PD 7–9, windows of exposure thought to differentially target the cerebellum and hippocampus. Sham-intubated and untreated rats served as controls. As juveniles, rats from each treatment condition were trained in either a long delay or trace eyeblink conditioning task. Alcohol-exposed rats demonstrated general conditioning impairments compared to controls during long delay conditioning, with more robust impairments in rats exposed to the higher alcohol dose (5.25 g/kg/day) than those that received the lower dose (4.66 g/kg/day). Alcohol-exposed rats showed trace conditioning impairments compared to controls only when the high dose of alcohol was administered over PD 4–9 or PD 7–9. These findings indicate significant learning and memory impairments following neonatal alcohol exposure at both PD 4–9 and PD 7–9. The pattern of impairments across delay and trace conditioning suggest that alcohol disrupts processes that are common to both tasks. These findings are consistent with studies of delay and trace eyeblink conditioning in children with FASD. Future studies of the mechanisms underlying these deficits will further our understanding of brain injury and memory impairments resulting from developmental alcohol exposure.

1. Introduction

Learning and memory impairments are consistently reported in children and adolescents with known prenatal exposure to alcohol [1–7]. In addition to a general reduction in cognitive function (e.g., IQ) [8], children with fetal alcohol spectrum disorders (FASD) show impairments on behavioral tasks that reflect specific domains of learning and memory. For example, children with FASD show significant acquisition deficits during classical eyeblink conditioning [3,4] that likely result from cerebellar abnormalities commonly demonstrated in this subject population [9,10]. Additionally, children with FASD show difficulty on spatial learning tasks [5–7],

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which may reflect abnormal hippocampal development following gestational alcohol exposure [7,11,12]. These behavioral and anatomical outcomes are also reported in animal models of FASD [13–16], which provide experimental control of the significant variables contributing to alcohol-related teratogenicity (e.g., the developmental timing and dose of alcohol exposure) [15–18]. The use of behavioral tasks like eyeblink conditioning in animal models of FASD may further our understanding of how the timing and dose of alcohol exposure contribute to domain-specific behavioral impairments.

Classical eyeblink conditioning offers a potential mechanism by which to examine both cerebellar and hippocampal function in the alcohol-exposed brain. During delay conditioning, a conditioning stimulus (CS; e.g., tone) overlaps and co-terminates with an unconditioned stimulus (US; e.g., airpuff to the eye or periocular shock). After repeated pairings, the previously neutral CS comes to elicit a conditioned response (CR; e.g., eyelid closure) prior to, or in the absence of, the US. The neural circuitry underlying delay eyeblink conditioning involves a brainstem-cerebellar pathway that is conserved across a number of species, including rodents and humans [19–24]. By imposing a stimulus-free "trace" interval between the CS and US, forebrain structures, such as the hippocampus, in addition to the underlying brainstem-cerebellar circuit, are needed for acquisition [25,26]. An additional distinction between delay and trace conditioning concerns the relative timing between CS and US onsets (the interstimulus interval [ISI]), which is usually longer during trace conditioning. By matching the ISI in delay (i.e., long delay) with that of trace conditioning, the behavioral ontogeny and acquisition rates are similar across tasks [27,28]; importantly, hippocampal lesions impair trace conditioning more than long-delay conditioning [25]. Long delay and trace eyeblink conditioning, therefore, provide an opportunity to examine both cerebellar and hippocampal function, respectively, in animal models of FASD.

The cerebellum and hippocampus are vulnerable to the neurotoxic effects of alcohol, especially when alcohol exposure is limited to the third-trimester equivalent in the rat (i.e., the first postnatal week) [15,16,29]. Purkinje cells within the cerebellum appear most vulnerable to the effects of alcohol prior to the 7th postnatal day (PD) [15,30], while alcohol exposure limited to either PD 4–9 or PD 7–9 results in CA1 pyramidal cell reduction within the hippocampus [14,16]. Limiting alcohol exposure to PD 7–9, therefore, is likely to result in significant hippocampal targeting relative to the cerebellum and may result in differential performance on long delay and trace eyeblink conditioning.

Recent studies in humans with FASD and in rodent models have utilized delay and trace eyeblink conditioning paradigms, demonstrating comparable conditioning impairments regardless of task [3,31]. Here we further explore the impact of developmental alcohol exposure on trace and long delay eyeblink conditioning in juvenile rats neonatally exposed to alcohol by manipulating the timing (PD 4–9 or PD 7–9) and dose (5.25 or 4.66 g/kg/day) of alcohol exposure. We hypothesized that rats exposed to alcohol over PD 4–9 would show impairments on both trace and long delay tasks, while those exposed over PD 7–9 would show greater impairments during trace than during long delay conditioning. We also hypothesized that greater deficits would appear in rats that received higher doses of alcohol.

2. Experiment 1A: the effects of a high binge dose of alcohol (5.25 g) over PD 4–9 on long delay and trace eyeblink conditioning

Neonatal alcohol exposure (PD 4–9) leads to cell loss in both the cerebellum and the hippocampus [15,16]. Here we ask what effects exposure to a high dose of alcohol (5.25 g/kg/day) over the neonatal period (PD 4–9) has on two task variants of eyeblink conditioning (EBC; trace and long delay) that both rely on a known cerebellar circuit but differ in their sensitivity to hippocampal disruption [19,25].

2.1. Materials and methods

2.1.1. Subjects

The subjects were 90 Long Evans rats (46 males and 44 females) from 29 litters bred at the University of Delaware. Breeder rats were housed overnight and if an ejaculatory plug was found the following morning that day was designated as gestational day (GD) 0. Pregnant females were housed in clear polypropylene cages (45 cm × 24 cm × 21 cm) with standard bedding and ad lib. access to water and rat chow. The date of birth was determined by checking for births during the light cycle (12:12) and, if newborn pups were found, that day was designated as postnatal day (PD) 0; all births occurred on GD 22. On PD 2, litters were transported from the breeding facility to be housed in the animal housing facility. Litters were culled to 8 pups (4 males and 4 females when possible) and received subcutaneous injections of a non-toxic black ink into one or more paws to aid in identification on PD 3. On PD 21, pups were weaned and housed with same-sex litter mates in 45 cm × 24 cm × 17 cm cages with ad lib. access to water and rat chow. On PD 24, rats were individually housed in small white polypropylene cages (24 cm × 18 cm × 13 cm) for the remainder of the study.

2.1.2. Alcohol dosing

Pups were exposed to a 5.25 g/kg/day single binge dose of alcohol (5.25 g), sham intubated (SI), or left undisturbed (UD) over PD 4–9 following procedures previously reported [32,33]. Within a given litter, pups were randomly assigned to receive alcohol or sham intubations. UD rats came from separate litters, which were left undisturbed with the exception that body weights were obtained on PD 4 and PD 9. Starting on PD 4, pups were briefly separated from their mothers and placed into Lexan containers placed over a heating pad (GE model #E12107) that was turned to the lowest setting to maintain body temperatures during separation. Alcohol was mixed with a custom milk formula [34] at a dose of or 5.25 g/kg/day (23.93% v/v). Pups were weighed each morning to determine the volume of solution to be administered at 0.0278 mL/g. Alcohol administration via intragastric intubation involved passing PE10 tubing lubricated with corn oil down the esophagus and into the stomach. The tubing was connected to a 1 mL syringe that was used to infuse the solution over a 15 s interval. Alcohol was administered in a single binge dose, followed 2 h later by an equal volume of milk-only solution. A second milk-only infusion was administered only on PD 4. Sham intubations involved an identical process with the exception that no solution was infused.

2.1.3. Blood alcohol concentration analysis

Two hours after alcohol/sham administration on PD 4, a 20 μL blood sample was collected from a small tail clip using a heparinized capillary tube. Blood samples from SI pups were disposed, while those collected from alcohol-exposed pups were centrifuged and plasma was collected and stored at −20 °C. Blood alcohol concentrations (BACs) were determined using an Analox GL5 Analyzer (Analox Instruments, Luneburg, MA) as previously described [17]. Briefly, the rate of oxidation of alcohol in each plasma sample was measured. BACs (expressed in mg/dl) were calculated based on comparisons to known values of an alcohol standard solution.

2.1.4. Eyeblink surgery

Subjects received eyeblink surgeries on PD 24 as previously described [17,35,36]. Pups were anesthetized with a ketamine/xylazine cocktail (87 mg/kg ketamine/13 mg/kg xylazine) with an intraperitoneal injection at a volume of 0.8 mL/kg. The scalp was cleaned with betadine and 70% isopropyl alcohol prior to incision and head stage implantation. The head stage consisted of differential electromyography (EMG) electrodes threaded through the upper left eyelid muscle, a bipolar stimulating electrode placed subdermally caudal to the left eye (to deliver the US), and a ground wire curled up and placed subdermally towards the back of the incision. The head stage was secured to the skull with dental acrylic that adhered to two 15 mm strips of galvanized steel wires implanted into the skull to act as anchors (placed in front and behind the head stage). Following surgery, an antiseptic opthalmic ointment was placed onto the eyes. The pups were then placed onto a heating pad set to the lowest temperature as they recovered from the anesthetic (~30–45 min).

2.1.5. Apparatus

Eyeblink conditioning occurred in one of 16 sound-attenuated conditioning chambers (BRS/LVE, Laurel, MD) as previously described [35,37]. Rats were placed into a stainless wire mesh cage within the conditioning chamber during testing. A fan provided background noise (70 dB). The auditory CS was produced by one of two speakers placed within each conditioning chamber. The current study used an 80 dB, 2.8 kHz tone presented for either 380 or 980 ms. The US was produced by a constant-current, 60 Hz square wave stimulator (World Precision Instruments, Sarasota, FL) set to deliver a 1.5 mA, 100 ms periocular shock. During conditioning sessions a wire lead that passed through the opening of the conditioning chambers was secured to