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Research report

Neonatal alcohol exposure impairs contextual fear conditioning in juvenile rats by disrupting cholinergic function

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HIGHLIGHTS

- Preexposure parameters influence the CPFE similarly in adult and juvenile rats.
- Enhanced preexposure does not alter impairment of the CPFE by neonatal alcohol.
- Neonatal alcohol impairs spatial cognition by disrupting cholinergic function.

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ABSTRACT

The context preexposure facilitation effect (CPFE) is a variant of context fear conditioning in which context preexposure facilitates conditioning to immediate foot shock. Learning about context (preexposure), associating the context with shock (training), and expression of context fear (testing) occur in successive phases of the protocol. The CPFE develops postnatally, depends on hippocampal NMDA receptor function, and is highly sensitive to neonatal alcohol exposure during the weanling/juvenile period of development [15,16]. The present study examined some behavioral and pharmacological mechanisms through which neonatal alcohol impairs the CPFE in juvenile rats. We found that a 5-min context preexposure plus five 1-min preexposures greatly increases the levels of conditioned freezing compared to a single 5-min exposure or to five 1-min preexposures (Experiment 1). Increasing conditioned freezing with the multiple- exposure CPFE protocol does not alter the neonatal alcohol-induced deficit in the CPFE (Experiment 2). Finally, systemic administration of 0.01 mg/kg physostigmine prior to all three phases of the CPFE reverses this ethanol-induced deficit. These findings show that impairment of the CPFE by neonatal alcohol is not confined to behavioral protocols that produce low levels of conditioned freezing. They also support recent evidence that this impairment reflects a disruption of cholinergic function [18].

1. Introduction

Alcohol is a major teratogen that damages the developing brain. Fetal Alcohol Syndrome (FAS) is the most common and preventable cause of intellectual and developmental disability, occurring in 0.2–7 cases per 1000 live births in the United States each year [1,2]. Fetal Alcohol Spectrum Disorder (FASD) is a broader term that characterizes children with prenatal alcohol exposure that have the developmental, behavioral, and cognitive deficits of FAS, but without the characteristic facial phenotype [2]. Children with FASD have behavioral impairments that include hyperactivity, and problems with attention, inhibition, motor performance, learning, and memory [3,4]. Impairments of brain and behavioral development found in the human disorder can be studied in animal models of FASD [2,3,5,6]. Research with animal models has contributed importantly to our understanding of the relationship between the pattern, timing, and dose of alcohol exposure, and subsequent neurobehavioral development.

The "brain growth spurt" is a period of extensive neurogenesis and synaptogenesis during the third trimester in humans when the brain is highly susceptible to the teratogenic effects of alcohol exposure [7,8]. Neonatal alcohol exposure is used as a rodent model of third trimester exposure in humans, as the brain growth

Abbreviations: EtOH, Ethanol exposed animals; BAC, Blood alcohol content; CF, continuous-five minute preexposure; CNC, Continuous + non-continuous preexposure; CPFE, Context preexposure facilitation effect; FAS, Fetal alcohol syndrome; FASD, Fetal alcohol spectrum disorder; GD, Gestational day; NC, Non-continuous preexposure; No Pre, No preexposure group; PD, Postnatal day; Phys, Physostigmine; Pre, Preexposure group; Sal, Saline; SI, Sham intubated animals; US, Unconditioned stimulus.

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spurt in the rat occurs around postnatal day (PD) 4–10 [7,9]. Alcohol exposure anytime during the brain growth spurt in the rat damages the hippocampus [8,10,11] and causes deficits on behavioral tasks that depend on the hippocampus [12–14]. Our lab has recently discovered that a variant of context fear conditioning, known as the context preexposure facilitation effect (CPFE) is especially sensitive to neonatal alcohol exposure compared to other commonly used tasks [14,15]. In the CPFE, learning about the context (preexposure), associating the context with the foot shock (training), and expressing contextual fear (testing) occur on three separate occasions, in which learned fear is only expressed if the animal is exposed to the testing context on the preexposure day. The basis for the sensitivity of the CPFE to neonatal alcohol is not fully understood. One possibility is that spatial learning of the context in the CPFE is "incidental" rather than "reinforcement-driven" [16]. For example, standard context conditioning, in which a context is encoded and associated with shock reinforcement on a single occasion, is less impaired by neonatal alcohol than is the CPFE, in which context encoding occurs incidentally (without shock reinforcement) on the day before the context-shock association is acquired [14]. Another possibility is that the CPFE is more sensitive to neonatal alcohol because it is merely a weaker form of context conditioning, involving lower levels of conditioned freezing than standard context conditioning. One goal of the present study was to test this possibility by re-examining the alcohol-induced deficit in the CPFE using a variant of the CPFE protocol that produces much higher level of conditioned freezing.

Another goal of this study was to test the hypothesis that neonatal alcohol impairs the CPFE by disrupting cholinergic function [17–19]. Cholinergic function in the hippocampus plays an important role in learning and memory, including contextual fear conditioning [20–22]. Recent work has shown that neonatal alcohol exposure disrupts the development of the cholinergic system [17,18] and that choline supplementation is capable of rescuing behavioral deficits caused by neonatal alcohol exposure [18,23]. The CPFE is an ideal behavioral paradigm for investigating the effects of cholinergic drugs on learning and memory because drug effects on different task components—context learning, fear conditioning, and expression of context fear—can be determined by administering drugs during the separate phases of the CPFE protocol [24,25]. The present study asked whether enhancing cholinergic function with physostigmine, an acetylcholinesterase inhibitor, would reverse the deficit in the CPFE shown by animals with neonatal alcohol exposure.

The present study consisted of three experiments. Experiment 1 determined whether the levels of contextual fear expressed in the CPFE can be increased by manipulating the preexposure protocol to include multiple exposures. A variant of the CPFE protocol involving multiple preexposures to the context enhances contextual fear conditioning in adult rats [26]. Experiment 1 sought to determine if the same is true for juvenile rats (PD31-33). Experiment 2 asked whether the sensitivity of the CPFE to neonatal alcohol is also seen with this variant of the CPFE protocol. Experiment 3 used this protocol to determine whether systemic administration of physostigmine can reverse the ethanol-induced deficit in contextual fear conditioning.

2. Experiment 1

Multiple context preexposures enhance the CPFE in adult rats [26–29] and mice [30], but it is not known whether this effect extends to developing rats. The present experiment therefore investigated the effect of multiple preexposures on the CPFE in juvenile rats. Rats were preexposed to the training and testing context, or to an alternate context. The preexposure protocol was manipulated such that rats were either exposed to the context for five 1-min exposures, or were preexposed to the context for 5 min with an additional five 1-min exposures. Performance of these groups was compared to previously published data involving a single 5-min preexposure. We predicted that the CPFE would be present in both protocols, however the protocol that included an additional five 1-min exposures would produce the highest levels of context fear conditioning.

2.1. Methods

2.1.1. Subjects

Subjects for Experiment 1 were 36 Long Evans rats (19 females and 17 males, derived from 5 litters bred at the Office of Laboratory Animal Medicine at the University of Delaware. Time-mated females were housed with breeder males overnight and were examined for an ejaculatory plug the following day and, if found, that day was designated as gestational day (GD) 0. Dams were housed in clear polypropylene cages measuring 45 cm × 24 cm × 21 cm with standard bedding and access to ad libitum water and rat chow. Animals were maintained on a 12:12 h light/dark cycle with lights on at 7:00 am. Date of birth was designated as postnatal day (PD) 0 (all births occurred on GD22). Litters were culled on PD3 to eight pups (usually 4 males and 4 females) and were paw-marked with subcutaneous injections of non-toxic black ink for identification. Pups were weaned from their mother on PD21 and housed with same-sex litter mates in 45 cm × 24 cm × 17 cm cages. On PD29 animals were individually housed in small white polypropylene cages (24 cm × 18 cm × 13 cm) with ad libitum access to water and rat chow for the remainder of the experiment. All subjects were treated in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Delaware following guidelines established by the National Institute of Health.

2.1.2. Apparatus and stimuli

Fear conditioning occurred in four clear Plexiglas chambers described previously [15]. They measured 16.5 cm × 12.1 cm × 21.6 cm and were arranged in a 2 × 2 formation on a Plexiglas stand within a fume hood which provided ambient light and background noise. Each chamber had a grid floor made of 9 stainless steel bars (11.5 cm from the top of the chamber), 0.5 cm in diameter and spaced 1.25 cm apart. The 2 second footshock unconditioned stimulus (US) was delivered using a shock scrambler (Med Associates, Georgia, VT ENV-414S) connected to the grid floor. Video of each session (preexposure, training, testing) was recorded using FreezeFrame software (Actimetrics, Wilmette IL), which measures change in pixilation, with freezing defined as a bout of 0.75 s or longer without a change in pixels. The FreezeFrame software recorded video from the four chambers simultaneously. The alternate context, *Context B*, was a wire mesh cage located in a different room in the same building. The cages used were the same chambers used for eye-blink conditioning, described in Brown & Stanton [16,31].

2.1.3. Design and procedure

Behavioral training occurred over three days from PD30-32 or PD31-33. Animals were assigned a priori to either the preexposure (Pre) or no preexposure (No Pre) group, and assigned to one of two different preexposure protocols, non-continuous preexposure (NC) or continuous + non-continuous preexposure (CNC). Animals in the preexposure group were preexposed to the training context (Context A), and those animals in the No Pre group were preexposed to the alternate context (Context B). Animals in the NC protocol were exposed to the context for one minute; they were then removed from the context, held in their transport cages in a nearby waiting room, and were returned 30–60 s later for a subsequent 1 min exposure. This was repeated for a total of five 1-min exposures to the context. Animals in the CNC protocol were first exposed to the context for five minutes and then removed. They were then returned to the context for five 1- minute exposures, as just described for the NC group. No more than one same-sex littermate was assigned to a given experimental group. Sex, preexposure, and protocol were equally represented in a given litter.

On the first day of the behavioral protocol, PD30 or 31, animals were weighed, and then placed in transport cages of clear Lexan (11 cm × 11 cm × 18 cm) covered with orange construction paper to obscure visual cues during transport. The rats were brought over and remained in an adjacent room to the testing room for <5 min, while the fear chambers were cleaned with 5% ammonium hydroxide solution. This weighing, cleaning, and transport protocol was consistent across all sessions and days. Pre animals were brought over and placed in Context A, which was the training and testing context described previously (see above: *Apparatus and stimuli*). Animals were preexposed to the context according to either the NC or CNC protocols (see above). Animals were then removed and returned to their home cage, ending the preexposure session. Animals in the No Pre group were preexposed with the NC, or CNC protocol to the alternate context (Context B).

Twenty-four hours later, animals from all groups were trained with an immediate (<5 s) 1.5 mA 2-s footshock in Context A. Rats were brought over one at a time, placed in their respective training chamber, and received an immediate footshock. Animals were immediately removed from the chamber following the