# PREFRONTAL MECHANISMS OF IMPAIRED COGNITION

# IN A RAT MODEL OF FASD

by

Nicholas A. Heroux

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychological and Brain Sciences

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| | |
|---|---|
| AAV | Adeno-associated virus |
| AC | Anterior cingulate |
| Alt-Pre | Rats preexposed to an alternative context |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AMY | amygdala |
| AP | anteroposterior |
| *Arc* | activity-regulated cytoskeleton-associated protein |
| ASO | antisense oligonucleotides |
| BAC | Blood alcohol concentration |
| BLA | Basolateral amygdala |
| CA1 | Cornu Ammonis area 1 |
| CA3 | Cornu Ammonis area 3 |
| CeA | Central nucleus of the amygdala |
| CFC | Contextual fear conditioning |
| CPFE | Context Preexposure Facilitation Effect |
| CREB | cAMP response element-binding protein |
| CS | Conditional stimulus |
| DG | Dentate gyrus |
| dHPC | Dorsal hippocampus |
| DNA | deoxyribonucleic acid |
| DV | dorsoventral |
| EB | Embryonic day |
| *Egr-1* | early growth response gene 1 |
| EPSP | Excitatory postsynaptic potentials |
| EtOH | Ethanol; Alcohol-exposed |
| FASD | Fetal Alcohol Spectrum Disorders |
| g | gram |
| GABA | gamma-aminobutyric acid |
| GD | Gestational day |
| HC | Home-cage controls (behaviorally naïve) |
| HPC | Hippocampus |
| hr | hour |
| IEG | Immediate early gene |
| IL | Infralimbic cortex |
| ISD | Immediate-shock deficit |
| kg | kilogram |
| LA | Lateral amygdala |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| ML | mediolateral |
| mm | millimeter |

| | |
|---|---|
| mPFC | Medial prefrontal cortex |
| mRNA | messenger ribonucleic acid |
| MUSC | Muscimol |
| NMDAR | N-methyl-D-aspartate receptor |
| *Npas4* | Neuronal PAS Domain Protein 4 |
| NR | Nucleus reunions |
| PAG | Periaqueductal gray matter |
| PBS | Phosphate buffered saline |
| PD | Postnatal day |
| PFC | Prefrontal cortex |
| PHR | Para-hippocampal region (rhinal cortices) |
| PHY | physostigmine |
| PL | Prelimbic cortex |
| Pre | Rats preexposed to the training context |
| qPCR | Quantitative real-time PCR |
| RNA | Ribonucleic acid |
| s | second |
| sCFC | Standard contextual fear conditioning |
| SCOP | Scopolamine |
| SI | Sham intubation |
| TFC | Trace fear conditioning |
| US | Unconditional stimulus |
| vHPC | Ventral hippocampus |
| VMT | Ventral midline thalamus |
| μg | microgram |
| μL | microliter |

## ABSTRACT

Fetal Alcohol Spectrum Disorders (FASDs) represent a significant medical and societal problem in the United States and abroad, representing a leading preventable cause of severe intellectual and developmental disability. Neurodevelopmental damage resulting from fetal alcohol exposure causes a severe disruption in prefrontal and hippocampal neuroanatomy and function, and thus causes pervasive cognitive impairments in learning and memory dependent upon these structures. While alcohol-induced disruptions in hippocampal anatomy and function have been extensively explored in both rodent models and humans, rodent work examining prefrontal mechanisms of impaired cognition is sparse. Accordingly, this dissertation characterizes the neurobiological mechanisms underlying context and contextual fear learning and memory and their impairment by third-trimester equivalent alcohol exposure in a rat model of FASD. This dissertation uses a variant of contextual fear conditioning (CFC) called the Context Preexposure Facilitation Effect (CPFE). In the CPFE, learning about the context, acquiring a context-shock association, and retrieving/expressing this association is temporally dissociated across three phases (context preexposure, immediate-shock training, and retention). Third-trimester equivalent exposure from postnatal day (PD) 4-9 abolishes retention test freezing in the CPFE in adolescent and adult rats (G. F. Hamilton et al., 2011; Murawski, Klintsova, & Stanton, 2012; Murawski & Stanton, 2010). Despite this, these previous studies are unable to dissociate PD4-9 alcohol effects on preexposure or training day processes because only retention test freezing was measured. In addition, while this

deficit was previously attributed to impaired hippocampal function, our lab has recently discovered that the medial prefrontal cortex (mPFC) is required during all three phases of the CPFE in adolescent rats (Heroux et al., 2017; Robinson-Drummer et al., 2017). This discovery largely motivated the experiments in this dissertation.

The first aim of this dissertation characterizes the effects of prefrontal (Experiment 6.1) or ventral hippocampal (Experiment 6.2) inactivation via local muscimol infusion during context exposure on expression of the immediate early genes (IEGs) *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC, dorsal hippocampal (dHPC), ventral hippocampus (vHPC), and ventral midline thalamus (VMT; consisting of reunions [NR] and rhomboid nuclei) in normally-developing adolescent rats. In Experiment 6.1 and 6.2, prefrontal or ventral hippocampal inactivation via muscimol infusion during context exposure abolished subsequent post-shock and retention test freezing in behaviorally-tested littermates of the sacrificed groups. In Experiment 6.1, we found that prefrontal inactivation impaired IEG expression in the mPFC, VMT, and vHPC but not dHPC during context preexposure. In Experiment 6.2, we found that ventral hippocampal inactivation during context preexposure disrupted IEG expression in the vHPC, mPFC, and dHPC but not VMT during context preexposure.

The second aim of this dissertation uses a rat model of FASD to characterize the effects of neonatal alcohol exposure from PD4-9 on regional neural activity and contextual learning and memory in the CPFE in adolescent rats. In Experiments 7.1 and 7.3, rat pups received oral intubation of alcohol (EtOH; 5.25 g/kg/day, split into two doses) or underwent sham-intubation (SI) from PD4-9 were tested on the CPFE from PD31-33. In Experiment 7.1, PD4-9 alcohol-exposed rats showed abolished post-shock and retention test freezing in the CPFE. In Experiment 7.2, alcohol-exposed rats

were unimpaired in standard contextual fear conditioning, in which context and context-shock learning occurs within the same trial. This task is "prefrontal-independent" but "hippocampal-dependent," defined by effects of loss-of-function manipulations in these structures. These data suggest that abolished post-shock freezing in the CPFE likely reflects disrupted prefrontal function supporting consolidation of the context representation. Accordingly, in Experiment 7.3, EtOH and SI rats were sacrificed 30 min after context preexposure and IEG expression in the mPFC and dHPC was analyzed via qPCR. Alcohol exposure impaired expression of the IEGs *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC but not dHPC during context exposure.

The third aim of this dissertation attempts to reverse these alcohol-induced neural and behavioral deficits by acute, pharmacological enhancement of cholinergic signaling during the CPFE. Our lab has shown that systemic administration of the acetylcholinesterase inhibitor physostigmine (PHY) prior to each phase rescues retention test freezing in PD7-9 alcohol-exposed rats (Dokovna, Jablonski, & Stanton, 2013). Whether this is also true of PD4-9 exposure and the exact phase of the CPFE that mediates this rescue and underlying neural mechanisms is unclear. Rats received oral intubation of alcohol (5.25g/kg/day) or SI from PD4-9, and then received a systemic injection of saline (SAL) or PHY (0.01mg/kg) prior to all three phases (Experiment 8.1.1) or just context exposure (Experiment 8.1.2) during the CPFE protocol from PD31-33. Administration of PHY prior to all three phases or just context preexposure rescued both post-shock and retention test freezing in EtOH rats without altering performance in SI rats. In Experiment 8.2, a subset of rats were sacrificed 30 min after context preexposure to assay changes in IEG expression in the

mPFC, dHPC, and vHPC. ETOH-SAL rats had significantly reduced mPFC but not dHPC expression of *c-Fos*, *Arc*, *Egr-1*, and *Npas4*. ETOH-PHY treatment rescued mPFC expression of *c-Fos* in alcohol-exposed rats and increased *Arc* and *Npas4* regardless of dosing condition. While there was no effect of PHY on dHPC or vHPC expression of *Arc*, *Egr-1*, or *Npas4*, this treatment significantly boosted hippocampal expression of *c-Fos* regardless of alcohol treatment.

The results of this dissertation have broad implications for basic behavioral neuroscience and FASD research. First, these studies were the first to suggest a role of mPFC-vHPC circuitry in incidental context learning and memory during the CPFE (Heroux, Horgan, Pinizzotto, Rosen, & Stanton, 2019). These findings shed further light on prefrontal involvement in contextual processes of Pavlovian contextual fear conditioning in normally- and abnormally-developing (e.g., alcohol-exposed) rodents. Second, these studies capture prefrontal dysfunction in a rat model of FASD, and highlight the importance of re-examining cognitive deficits resulting from developmental alcohol exposure that historically has been attributed solely to hippocampal dysfunction. Finally, these studies demonstrate the efficacy of acute treatment with drugs that enhance cholinergic signaling in reversing neural and cognitive impairments seen in a rat model of FASD. These studies provide a foundation for future work examining the efficacy of similar treatments in other models of FASD and in humans.

# Chapter 1

# INTRODUCTION

## 1.1 The *Context Preexposure Facilitation Effect*

This dissertation uses a variant of contextual fear conditioning (CFC), called the *Context Preexposure Facilitation Effect* (CPFE), to elucidate the neural mechanisms of contextual learning and memory and how these processes are impaired by developmental alcohol exposure in a rodent model of Fetal Alcohol Spectrum Disorder (FASD). The CPFE differs from standard contextual fear conditioning (sCFC), in which rats learn about the context and acquire a context-shock association within the same training session (see **Figure 1.1**). In the CPFE, learning about the context, acquiring a context-shock association, and retrieval/expression of this association is separated across three days (context preexposure, immediate-shock training, and retention; see **Figure 1.2**). The CPFE depends on the encoding of contextual cues on the preexposure day that are subsequently consolidated into a conjunctive context representation (Pre group in Figure 1.2; Jablonski, Schiffino, & Stanton, 2012; Rudy & O'Reilly, 1999). During immediate-shock training, hippocampal-dependent pattern completion allows this retrieved conjunctive representation to be associated with immediate foot-shock (Rudy, 2009). Acquisition of this context-shock association can be probed in a post-shock freezing test immediately after context-shock pairing on the training day (Jablonski et al., 2012). Successful retention freezing test performance twenty-four hours later reflects consolidation and retrieval of the context-shock association. Rats preexposed to an

alternate context on the first day (Alt-Pre group in Figure 1.2) demonstrate the immediate-shock deficit, which reflects an inability to form a context-shock association with insufficient exposure to the training context (Fanselow, 1990). The CPFE emerges between postnatal day (PD) 17 and PD24 and is dependent on activity in dorsal (dHPC) and ventral (vHPC) hippocampus during all three phases in rats (Cullen, Ferrara, Pullins, & Helmstetter, 2017; Jablonski et al., 2012; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Rudy & Matus-Amat, 2005; Schiffino, Murawski, Rosen, & Stanton, 2011). Our lab has recently discovered that inactivation of the medial prefrontal cortex (mPFC) during any phase disrupts the CPFE in adolescent rats (Heroux, Robinson-Drummer, Sanders, Rosen, & Stanton, 2017; Robinson-Drummer, Heroux, & Stanton, 2017). While these data indicate a novel role of this structure in contextual learning and memory during incidental context exposure, prefrontal mechanisms supporting this behavior are unclear. We hypothesized that prefrontal inactivation disrupts hippocampal activity recruited for the formation of a long-term context representation via their reciprocal connectivity with the ventral midline thalamus (VMT; consisting of reunions [NR] and rhomboid nuclei). Accordingly, the first aim of this dissertation asked the question: *Does prefrontal inactivation during incidental context exposure effect molecular activity in the ventral midline thalamus and hippocampus?* This question is addressed by examining the effects of prefrontal inactivation on immediate early gene (IEG) expression in the mPFC, dHPC, and VMT during context preexposure. Given that this inactivation impaired gene expression in the vHPC and VMT but not the dHPC, we performed a second experiment that asked: *Does ventral hippocampal inactivation during incidental context exposure impair IEG expression in the prefrontal cortex?*

Collectively, these studies further characterize circuitry underlying incidental context learning and memory, which can then inform deficits that we observe in subsequent aims directed at our rodent model of FASD. The results of the first aim are presented in **Chapter 6**.

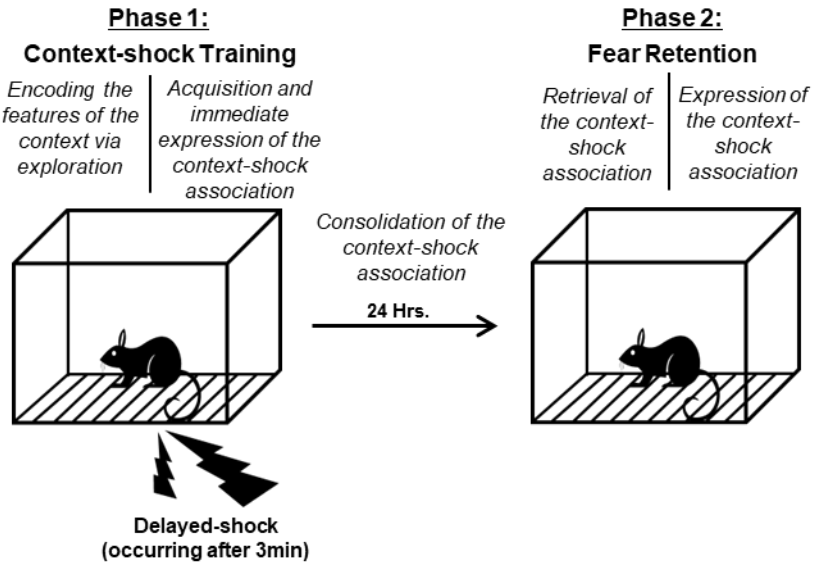


Figure 1.1 Schematic representation of standard contextual fear conditioning (sCFC) and associated learning and memory processes during each phase. Rats receive 3min of novel context exposure before receiving two foot-shocks, in the absence of any discrete cues. This association can be probed in a post-shock freezing test immediately after shock presentation as a measure of acquisition, or retrieved and expressed in a retention freezing test twenty-four hours later. A group that receives shock immediately rather than after a 3-min delay is used to control for non-associative aspects of the training experience. Image adapted from Jablonski et al. (2014).

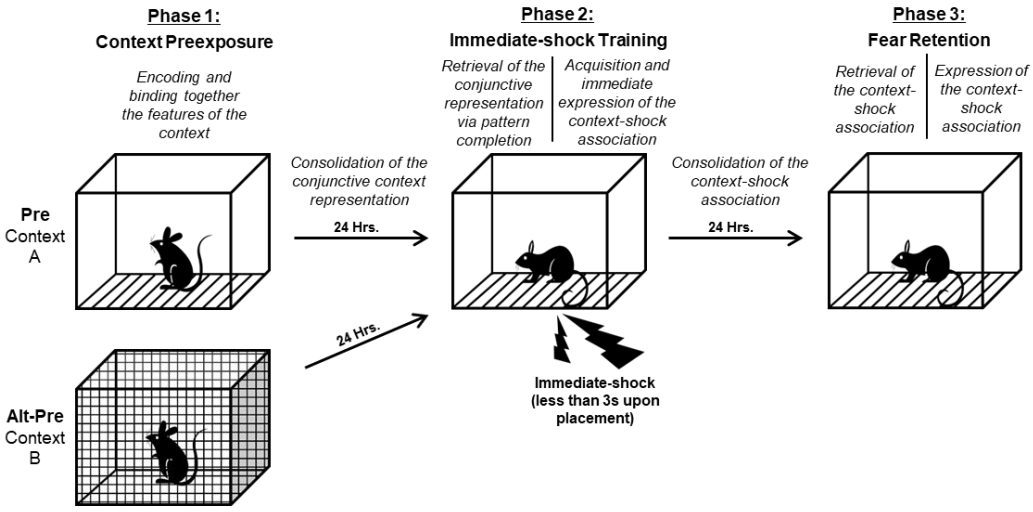


Figure 1.2 Schematic representation of the *Context Preexposure Facilitation Effect* (CPFE) and associated learning and memory processes during each phase. During the first phase ("Context Preexposure"), rat receive context exposure in the absence of any aversive stimuli, in which they encode features of the context that are subsequently bound into a conjunctive context representation. Rats in the Pre group receive context exposure to the context in which immediate-shock training later occurs (Context A), whereas rats in the Alt-Pre group receive exposure to an alternative context (Context B). During the second phase ("Immediate-shock Training"), both Pre and Alt-Pre rats receive immediate shock(s) in Context A occurring less than 3s upon chamber entry. Rats in the Pre group undergo rapid pattern completion to retrieve the conjunctive context representation to associate with shock. Rats in the Alt-Pre group are unable to form a context-shock association without prior exposure to the training context (also called the immediate-shock deficit; Fanselow, 1990). This association can be probed in a post-shock freezing test, or retrieved and expressed in a retention freezing test twenty-four hours later ("Fear Retention"). The CPFE reflects a significant elevation in freezing in the Pre group above the Alt-Pre group. Image adapted from Jablonski et al. (2014).

## 1.2 Developmental Alcohol Exposure and the CPFE

Neurocognitive impairment resulting from gestational alcohol exposure is a preventable but leading cause of severe intellectual and developmental disability, with an estimated prevalence as high as 5% across diverse communities in the United States (May et al., 2018, 2009; Murawski, Moore, Thomas, & Riley, 2015). Alcohol acts as a teratogen in the developing nervous system, disrupting limbic system development and cognition dependent on brain structures such as the hippocampus and prefrontal cortex (Murawski et al., 2015; Norman, Crocker, Mattson, & Riley, 2009; Wozniak et al., 2016). Hippocampal deficits are captured by rodent models of FASD using third-trimester equivalent exposure, in which rats receive neonatal alcohol exposure via intragastric intubation during the brain growth spurt (i.e., occurring during the first ten days of life in the rat; Marino, Aksenov, & Kelly, 2004; Murawski, Klintsova, & Stanton, 2012; Patten, Fontaine, & Christie, 2014; Thomas, Wasserman, West, & Goodlett, 1996; Tran & Kelly, 2003). *One major goal of this dissertation is to elucidate the behavioral and neurobiological mechanisms by which this neonatal alcohol exposure impairs cognition in rats.* Of particular interest is the role of prefrontal dysfunction, which, despite being an emerging hallmark of the human condition, has largely been ignored in animal model research focusing on hippocampal dysfunction.

The CPFE is particularly sensitive to the effects of developmental alcohol exposure in rats. Neonatal alcohol exposure from PD4-9 abolishes the CPFE but leaves sCFC and cued fear conditioning intact (G. F. Hamilton et al., 2011; Murawski et al., 2012; Murawski & Stanton, 2010, 2011). This impairment of the CPFE linearly scales with alcohol dose, with a significant negative correlation of blood-alcohol concentrations during alcohol exposure with retention test performance (Murawski &

Stanton, 2011). This exposure also results in a specific knockdown of hippocampal *c-Fos* expression that cannot fully be attributed to hippocampal CA1 pyramidal cell loss on the preexposure day of the CPFE in adolescent rats (Murawski et al., 2012). Despite these studies, the phase of the CPFE that mediates cognitive impairment produced by PD4-9 alcohol exposure remains unclear because only retention test freezing was measured. Given our recent discovery of prefrontal dependence of preexposure day processes of the CPFE (Heroux et al., 2017), the role of prefrontal dysfunction in this alcohol-induced behavioral deficit is also of great interest. Accordingly, the second aim of this dissertation asks two questions: (1) *Does neonatal alcohol exposure disrupt learning and memory processes associated with context preexposure or immediate-shock training in the CPFE?*, and (2) *How does neonatal alcohol exposure effect molecular activity in the prefrontal cortex and hippocampus during the phase mediating behavioral disruption in the CPFE?* The first question is addressed by examining the effects of PD4-9 alcohol exposure on both post-shock and retention test freezing in the CPFE, thereby adding an earlier behavioral outcome measure on the training day (Jablonski & Stanton, 2014). Given that alcohol-exposed rats can learn about the context and acquire a context-shock association in sCFC (Murawski & Stanton, 2010; see **Chapter 7**), any disruptions observed in post-shock freezing likely reflect disrupted consolidation or retrieval of the context representation in the CPFE. The second question is addressed by measuring IEG expression in the HPC and PFC during the phase in which alcohol-exposed rats are impaired. These results are presented in **Chapter 7**. Finally, once discovering the phase of the CPFE that mediates alcohol-induced cognitive impairment, the third aim of this dissertation asks the question: *Can these disruptions be rescued by enhancing cholinergic*

*signaling via acute, pre-phase acetylcholinesterase inhibitor treatment?* Emerging evidence suggests that cholinergic dysfunction represents a substantial mechanism by which alcohol disrupts neuronal development and cognition across the lifespan (see **Chapter 3** for detailed discussion). Our lab has shown that systemic administration of an acetylcholinesterase inhibitor prior to all three phases recues in CPFE in rats receiving PD7-9 alcohol exposure (Dokovna et al., 2013). The exact phase-dependent cognitive processes and neural correlates this treatment rescues and whether this would generalize to the PD4-9 window is unclear. The final aim of this dissertation addresses this question and the results are presented in **Chapter 8**.

## 1.3 Dissertation Overview

This dissertation is organized into three sections. The first section consists of **Chapters 2-4**. It provides background information on the neurobiology of Pavlovian contextual fear conditioning (**Chapter 2),** rodent models of FASDs (**Chapter 3**), and previously published work from our lab that is foundational for the experiments in this dissertation (**Chapter 4**). The second section consists of **Chapters 5-8**. It details the general methods and materials common across dissertation experiments (**Chapter 5**) followed by an overview, analysis, and interpretation of each dissertation aim adapted from published manuscripts (**Chapters 6-8**). The third section includes a summary of experimental findings, general conclusions, and future directions relating to the aims of this dissertation (**Chapter 9**).

# Chapter 2

# NEUROBIOLOGY OF CONTEXTUAL FEAR CONDITIONING

The preceding chapter described the structure and aims of this dissertation, and introduced sCFC and CPFE behavioral paradigms. Using the CPFE, this dissertation examines the neurobiological and behavioral mechanisms of context and contextual fear learning and memory in normally and abnormally (i.e., alcohol-exposed) developed adolescent rats. The current chapter provides a brief overview of the involvement of HPC and PFC in sCFC and background conditioning occurring during auditory and trace fear conditioning. This chapter also introduces the study of IEGs as molecular markers of regional neural activity and plasticity during discrete phases of behavioral tasks in rodents.

## 2.1 Neuroanatomical Substrates of Contextual Fear Conditioning in Rodents

It is a widely accepted view that multiple neural systems support distinct processes of learning and memory in Pavlovian contextual fear conditioning (Fanselow & Poulos, 2005). CFC involves learning an association between a neutral context a rat is exploring (or has explored previously) and aversive foot-shock(s) that occurs in this context. The context can be defined as a set of stable, multi-modal (spatial, olfactory, auditory, interoceptive, etc.) features that are distinct from more transient and salient discrete cues occurring within the context (Maren, Phan, & Liberzon, 2013; Rudy, 2009). The foot-shock(s) serves as the unconditional stimulus (US), and the context serves as the conditional stimulus (CS) that elicits a species-

typical freezing response that is one of many responses that reflect a central state of conditioned fear in rodents (Davis, 1992; Fendt & Fanselow, 1999). Early models of the neurobiology of CFC in rodents focused on a circuit involving coordinated communication between the thalamus, para-hippocampal region (PHR; consisting of the rhinal cortices), HPC, basolateral amygdala (BLA), and midbrain output structures (Anagnostaras, Gale, & Fanselow, 2001; Fanselow & Poulos, 2005; Fendt & Fanselow, 1999; Maren, 2001; Phillips & LeDoux, 1992; see **Figure 2.1**). In these models, sensory information about the context CS and the foot-shock US activate thalamic nuclei, after which US information is sent directly to the BLA, whereas CS information is sent to the PHR and HPC. These latter regions were thought to encode features of the context and form a context representation (Anagnostaras et al., 2001; Rudy, 2009), and this information is propagated to the BLA, where the context-shock association is formed (Fendt & Fanselow, 1999). The BLA then projects to the central nucleus of the amygdala (CeA), which drives conditioned autonomic arousal and species-typical freezing responses via efferent projections to the hypothalamus/brain stem and periaqueductal gray (PAG), respectively (Fendt & Fanselow, 1999). While this basic framework has received much empirical support, these early models did not include any role of prefrontal neuronal circuitry in processes of CFC. Recent models have suggested roles of the mPFC in attentional processing, consolidation, reconsolidation, contextual control, and generalization of fear memories (Fanselow & Poulos, 2005; Gilmartin, Balderston, & Helmstetter, 2014; Giustino & Maren, 2015; Rozeske, Valerio, Chaudun, & Herry, 2015). In the following section, I include an overview of research characterizing both hippocampal and prefrontal involvement in acquisition and retention of CFC and related behavioral paradigms.

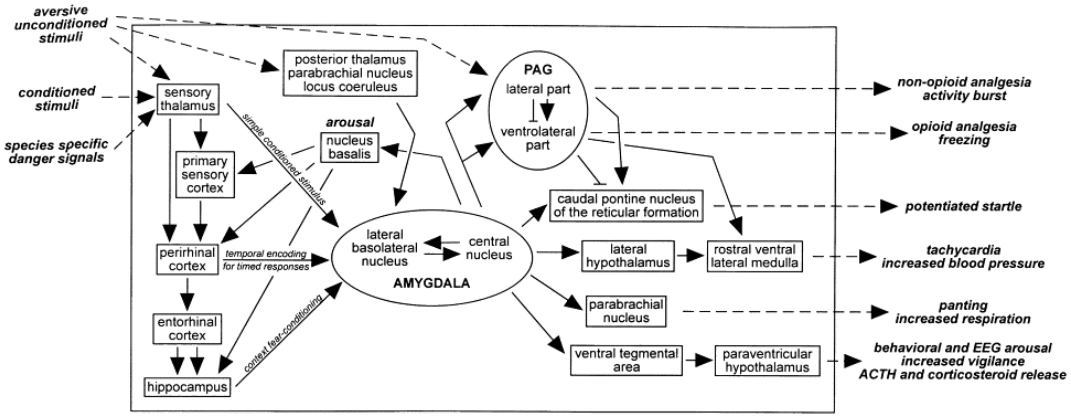


Figure 2.1 Hypothetical circuit mediating the different aspects of conditioned fear. Abbreviations: ACTH, adrenocorticotrophic hormone; EEG, electro-encephalogram; PAG, periaqueductal gray. Taken from Fendt & Fanselow (1999).

### 2.1.1 Hippocampal Involvement in Acquisition and Retention of Contextual Fear Conditioning

The hippocampus has long been thought to underlie contextual learning and memory across variants of Pavlovian fear conditioning (Fanselow, 2010; Fanselow & Poulos, 2005; Fendt & Fanselow, 1999; Maren et al., 2013; Rudy & O'Reilly, 1999b). The dual-process theory posits that contextual fear learning can be supported by two different associative systems: a neocortical elemental system or a hippocampal configural system (Anagnostaras, Gale, & Fanselow, 2001; Maren, 2001; O'Reilly & Rudy, 2001; Rudy, 2009; Rudy & O'Reilly, 1999a; see **Figure 2.2A**). In the elemental system, individual features of the context enter into an independent association with foot-shock and their additive strength determines conditioned fear. In the configural system, features of the context are bound (non-additively) into a conjunctive representation that is associated with foot-shock. Biological models of this theory suggested that elemental conditioning occurs via direct projections from the PHR and sensory cortex to the BLA, in which elemental CS-US associations are supported by amygdalar plasticity in the absence of higher cortical processing (Maren, 2001; see **Figure 2.2B**). In contrast, configural conditioning occurs via sensory information about context features converging in the HPC, in which features are bound into a conjunctive representation by hippocampal plasticity before being sent to the amygdala (AMY; see **Figure 2.2B**). Evidence from lesion studies suggests that the default system that supports contextual fear conditioning is the hippocampal system (Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006). For example, Maren et al. (1997) and Wiltgen et al. (2006) demonstrated that retrograde (post-training) but not anterograde (pre-training) lesions of the HPC disrupt retention test freezing during sCFC in rats (see **Figure 2.3**). These data indicate that sCFC does not require the

HPC, but if intact, the HPC supports contextual fear learning and overshadows the elemental feature-based system. Importantly, rats with anterograde lesions required two additional trials and longer placement-to-shock intervals to acquire a context-shock association, which indicates the elemental system takes additional training and is slower than the more rapid hippocampal system (Wiltgen et al., 2006). Numerous other studies have supported hippocampal system dominance, as pre-training lesions or reversible inactivation of either the dHPC or vHPC leave CFC intact, while post-training manipulations produce severe retrograde amnesia (Ballesteros, De Oliveira Galvão, Maisonette, & Landeira-Fernandez, 2014; Paul W. Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Hunsaker & Kesner, 2008; J. J. Kim, Rison, & Fanselow, 1993; J. Q. Lee, Sutherland, & McDonald, 2017; Maren, Aharonov, & Fanselow, 1997; Maren & Holt, 2004; Matus-Amat et al., 2004; Wiltgen et al., 2006; W.-N. Zhang, Bast, Xu, & Feldon, 2014). In contrast, pre-training NMDA receptor (NMDAR) antagonism in the dHPC or vHPC disrupts contextual fear retention but leaves post-shock freezing intact (Czerniawski et al., 2011; J. J. Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; J. J. Kim, Fanselow, DeCola, & Landeira-Fernandez, 1992; Quinn, Loya, Ma, & Fanselow, 2005; Sanders & Fanselow, 2003; Tayler et al., 2011). These data indicate that compensation occurs after HPC lesion or inactivation, but in an otherwise intact rat, consolidation of long-term contextual fear memories during sCFC requires dHPC and vHPC plasticity. It's also important to note that these previous lesion and reversible inactivation studies do not show whether conditioning was elemental vs. configural, so it's possible that, in the absence of the HPC, the neocortical system also supports configural learning but is slower than the hippocampal system (i.e., requires additional training; Fanselow, 2010). This notion is

supported by the finding that HPC-lesioned rats still benefit from context preexposure and show a (weak) CPFE (Rudy, Barrientos, & O'Reilly, 2002; see **Figure 2.4**), which can only be supported by conjunctive but not feature-based context representations (see **Chapter 1**; Fanselow, 2010; Jablonski, Schiffino, & Stanton, 2012; Rudy & O'Reilly, 1999b). Additional experiments are needed to test the parameters under which learning in the absence of the HPC is elemental vs. configural across variants of CFC. Nevertheless, this research establishes a key role of the configural HPC system in supporting CFC in rodents.

Recent experiments using gain- and loss-of-function chemogenetic and optogenetic approaches have supported these earlier studies and provided insights into hippocampal mechanisms of context and contextual fear memory (Asok, Leroy, Rayman, & Kandel, 2019; Tonegawa, Liu, Ramirez, & Redondo, 2015). These experiments have used activity-dependent neuronal tagging in transgenic mice to visualize and later manipulate neurons expressing the IEGs *c-Fos* and *Arc* in discrete brain regions during training in foreground and background CFC (see Tonegawa et al., 2015 for review). Early loss-of-function studies using this technology demonstrated that, during memory recall, optogenetic inactivation (via labeled *ArchT*) of hippocampal CA1, CA3, or DG neurons tagged during training impairs retention test freezing in sCFC (Denny et al., 2014; Tanaka et al., 2014). These studies showed that hippocampal neurons activated by training participate in the long-term memory trace (or "engram"), and that these neurons are necessary for successful memory recall. Importantly, these studies cannot conclude that the hippocampus is the site of the engram or memory storage, in part because this likely occurs across a distributed network. Accordingly, Tanaka et al. (2014) demonstrated that optogenetic silencing of

"engram" neurons in CA1 selectively prevented activation of "engram" neurons in other brain regions such as the rhinal cortices and amygdala without significantly altering total activity in these regions. Gain-of-function studies showed that reactivation of hippocampal neurons active during acquisition is sufficient to elicit freezing during a retention test in a non-conditioned context (see **Figure 2.5A**; Liu et al., 2012; Tonegawa et al., 2015). Ramirez et al. (2013) and Garner et al. (2012) demonstrated that hippocampal or whole-brain activation of neurons encoding a novel context (A), when reactivated during fear conditioning in a different context (B), form a context-shock association to the former (A) despite never receiving foot-shock in this context (see **Figure 2.5B**). Another study showed that concurrent reactivation of HPC neurons encoding context and AMY neurons encoding immediate shock in the home-cage is sufficient to form an artificial context-shock association in the absence of training (Ohkawa et al., 2015). Additional studies have shown that endogenous and experience-driven excitability (via varying levels of signaling molecules such as cAMP response element-binding protein [CREB]) in neurons largely determines their allocation to "engrams" or memory traces at the time of behavioral experience (Cai et al., 2016; J. Han et al., 2007; Josselyn & Frankland, 2018). One important caveat for this optogenetic research is that behavioral change or expression caused by artificial stimulation or inhibition may not accurately represent what occurs naturally within a memory system; moreover, state-dependency issues also emerge in loss-of-function experiments as light is typically only turned on during one phase of the behavioral paradigm. Nevertheless, in conjunction with previous lesion and pharmacological studies, this research establishes a key role of the HPC in contextual processes of CFC in rodents.

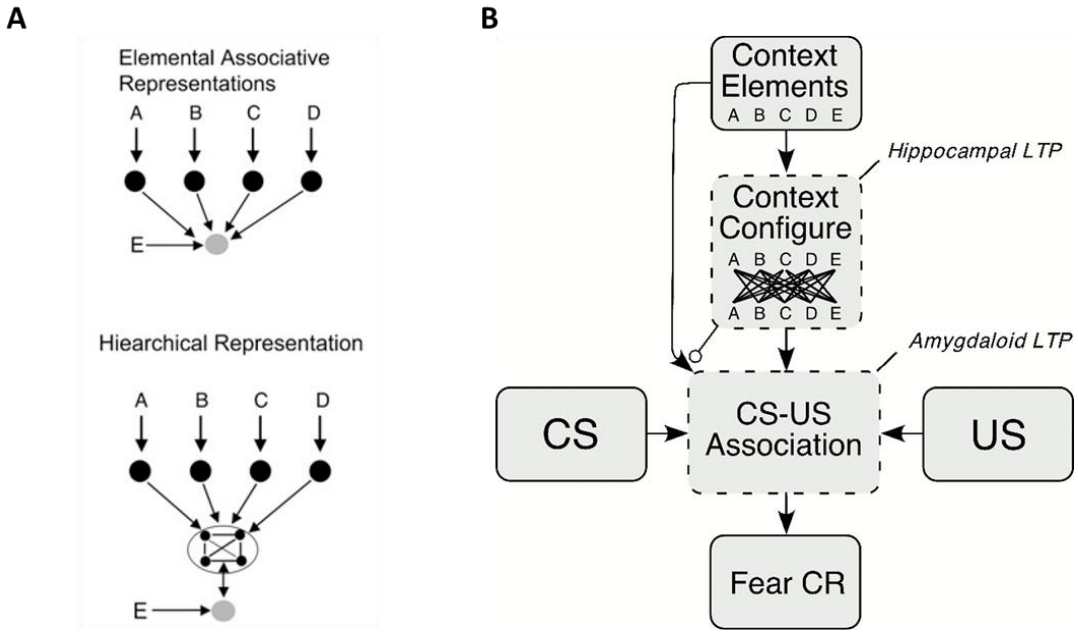


Figure 2.2 Psychological **(A)** and neurobiological **(B)** processes of the dual-process theory of Pavlovian contextual fear conditioning. (**A)** In the elemental system (top), features of the context (A, B, C, D) enter into independent associations with the foot-shock US (E). In the configural system (bottom), features of the context are bound into a conjunctive representation and this representation is associated with foot-shock. **(B)** In the elemental system, features of the context are sent directly to the amygdala where the context-shock association is formed via amygdalar plasticity. In the configural system, these features are bound into a conjunctive representation by hippocampal plasticity before being sent to the amygdala. Taken and adapted from Rudy (2009) and Maren (2001).

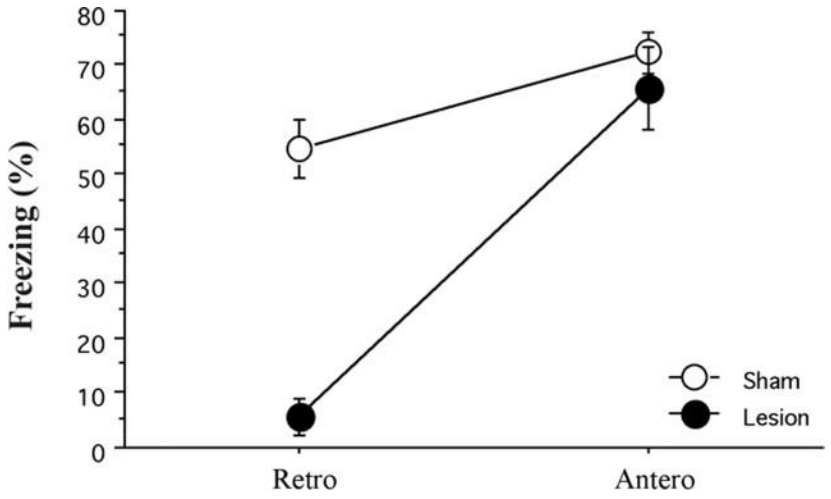


Figure 2.3 Effects of pre-training (anterograde) vs. post-training (retrograde) lesions of the hippocampus on retention test freezing in sCFC. Retrograde but not anterograde lesions of the hippocampus abolish retention test freezing relative to sham controls. Taken and adapted from Wiltgen et al. (2006).

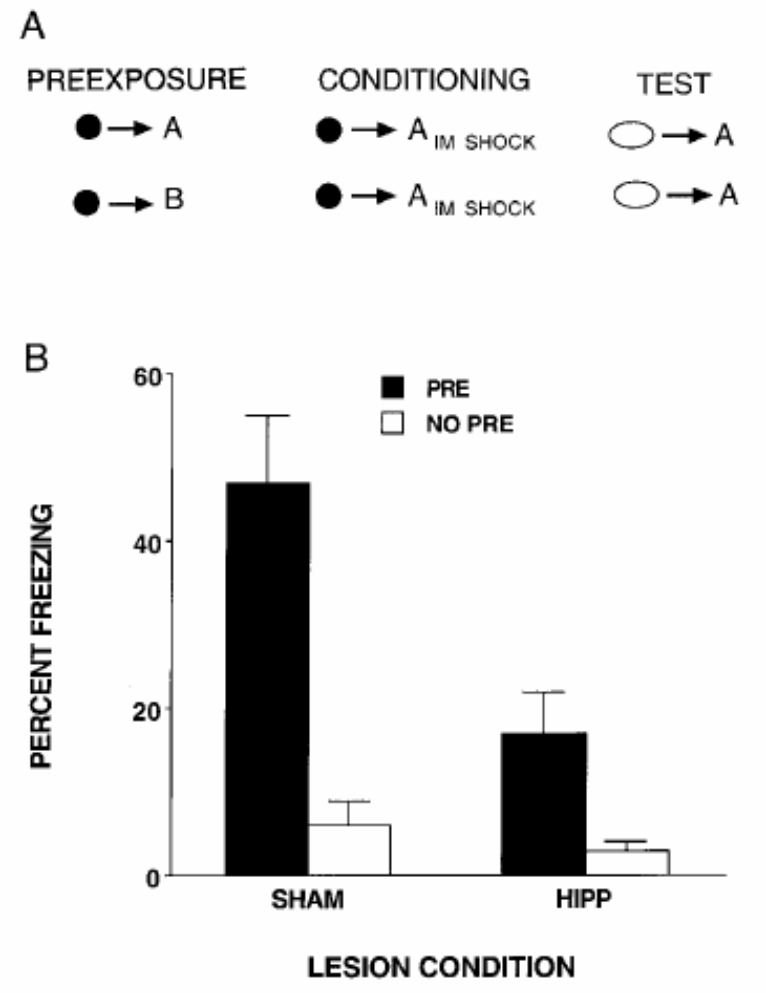


Figure 2.4 Effects of anterograde HPC lesions on retention test freezing in the CPFE. **(A)** Rats were preexposed to the training context (A; Pre) or an alternate context (B; No Pre) before receiving immediate-shock and retention testing in Context A. **(B)** Both sham control and lesioned rats show a significant CPFE (Pre>No Pre), but lesioned rats are significantly impaired relative to sham rats. Taken and adapted from Rudy et al. (2002).

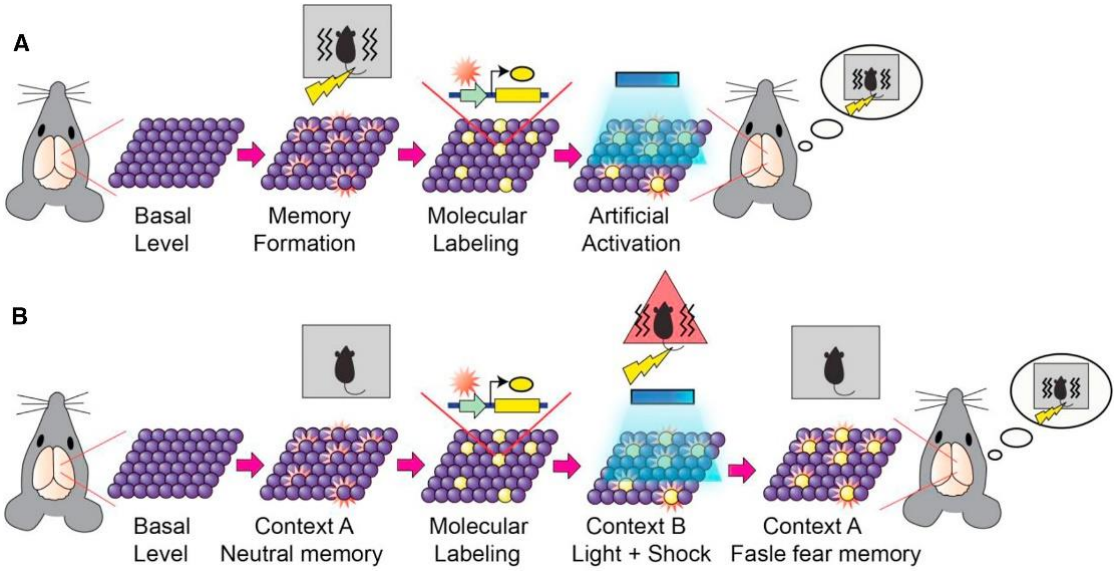


Figure 2.5 Optogenetic manipulations of memory engram cell populations. **(A)** Light activation of memory engram cell population caused memory recall. Neurons active during the formation of a contextual fear memory were labeled by ChR2. When these neurons were artificially activated by light stimulation in a different context, the animals displayed freezing behavior, suggesting the recall of the previous context associated with fear. **(B)** Generation of a false contextual fear memory. Neurons active in a neutral context were labeled with ChR2 and later reactivated by light in a different context while the animals simultaneously received foot shock. When the animals were returned to the original neutral context, they displayed fear response, suggesting the recall of a false memory associating the neutral context and the foot shock. Taken from Tonegawa et al. (2015).

### 2.1.2 Prefrontal Involvement in Acquisition and Retention of Contextual Fear Conditioning

The role of PFC in the regulation of fear expression and extinction learning and memory is extensively studied and well known. This topic is not reviewed here as it is less relevant and has been extensively reviewed elsewhere (Giustino & Maren, 2015; Maren et al., 2013; Tovote, Fadok, & Luthi, 2015). However, a growing body of work has also implicated the prefrontal cortex as an important brain region supporting contextual learning and memory processes across variants of CFC (see reviews: Gilmartin et al., 2014; Giustino & Maren, 2015; Rozeske et al., 2015). Early models of CFC (see **Figure 2.1**) did not include the PFC because lesions to the dorsal or ventral axis (consisting of anterior cingulate [AC] / prelimbic cortex [PL], or infralimbic cortex [IL]) did not impair acquisition or retention of cued or contextual fear conditioning in rats (Gewirtz, Falls, & Davis, 1997; Holson & Walker, 1986; Lacroix, Spinelli, Heidbreder, & Feldon, 2000; M. A. Morgan & LeDoux, 1995; M. A. Morgan, Romanski, & LeDoux, 1993; Quirk, Russo, Barron, & Lebron, 2000). Pre-training inactivation of whole mPFC or individual sub-regions also has no effect on sCFC, but inactivation prior to testing generally impairs contextual fear expression (Corcoran & Quirk, 2007; C. J. Han et al., 2003; Heroux et al., 2017; Sierra-Mercado, Corcoran, Lebrón-Milad, & Quirk, 2006; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011). These studies led to a dogma that the mPFC is generally not required for the acquisition of simple CS-US associations, but instead regulates the expression of previously learned associations during sCFC (Gilmartin et al., 2014; Sotres-Bayon & Quirk, 2010). However, as seen after HPC insult, compensation by other brain regions can occur after lesions or reversible inactivation, which means that lack of an effect does not mean that the brain region isn't involved in an otherwise "intact" animal (see

Biedenkapp & Rudy, 2009; Coelho, Ferreira, Kramer-Soares, Sato, & Oliveira, 2018; Wiltgen et al., 2006; Zelikowsky et al., 2013). Accordingly, several studies have shown a role of plasticity within the AC sub-region of the mPFC in supporting the consolidation but not acquisition of context-shock associations in rats (Einarsson & Nader, 2012; Rozeske et al., 2015; Vetere et al., 2011; Zhao et al., 2005). For example, pre-training NMDAR antagonism or post-training protein synthesis inhibition (via anisomycin) impairs retention test freezing (see **Figure 2.6**; Einarsson & Nader, 2012). Consistent with disruptions caused by anisomycin, post-training blockade of dendritic spine growth in pyramidal neurons in the AC also impairs retention test freezing (Vetere et al., 2011). Interestingly, reversibly inactivating the AC but not PL during training impairs retention test freezing (Corcoran & Quirk, 2007; Tang et al., 2005). One common result is that disruptions caused by AC insult are relatively small, with animals still freezing well above (>30%) what a non-associative control would likely freeze (if included). Regardless, these studies suggest a modulatory role of the AC in the consolidation of newly formed context-shock associations during sCFC (Rozeske et al., 2015). Other pharmacological studies show that acquisition of background contextual fear during auditory-delay and trace fear conditioning (TFC) procedures is impaired by prefrontal inactivation, NMDA-receptor antagonism, or protein synthesis inhibition (Gilmartin & Helmstetter, 2010; Gilmartin, Kwapis, & Helmstetter, 2012, 2013; Gilmartin, Miyawaki, Helmstetter, & Diba, 2013). In addition, acquisition and retention of TFC is highly sensitive to prefrontal insult, in part because sustained firing via prefrontal NR2B-containing NMDARs and muscarinic receptor activity is thought to underlie “bridging” of the CS and US during the trace interval (Beeman, Bauer, Pierson, & Quinn, 2013; Gilmartin et al., 2014;

Gilmartin, Kwapis, et al., 2013; Zhao et al., 2005). Moreover, in the CPFE, separation of context vs. context-shock learning necessitates activity and cholinergic function in the mPFC (see **Chapter 4**; Heroux, Robinson-Drummer, Sanders, Rosen, & Stanton, 2017; Robinson-Drummer, Heroux, & Stanton, 2017). Collectively, these studies suggest prefrontal involvement in 1) plasticity underlying consolidation of newly formed context-shock associations (Rozeske et al., 2015), and 2) CFC procedures where contextual learning is less salient (e.g., in background conditioning with discrete cues present) or when there is temporal separation of component processes (e.g., in trace fear conditioning or the CPFE; see Heroux et al., 2017; Gilmartin et al., 2014).

Armed with these discoveries, Rozeske et al. (2015) proposed a new model of the acquisition of CFC that included the mPFC (see **Figure 2.7;** red lines are supported by data, gray lines are hypothetical). In this model, contextual inputs arrive in the HPC where a context representation is formed and converges with foot-shock US inputs into the BLA to support formation of a context-shock association. The AC receives input from both the HPC and BLA and modulates consolidation of the context-shock association within this circuit. The AC can also drive fear expression via direct connectivity with the AMY and PAG. Recent studies silencing this PFC-PAG pathway revealed its importance for the contextual specificity of conditioned freezing, expanding early models that labeled PAG as solely an output structure (Herry & Johansen, 2014; Rozeske et al., 2018). Based on recent data (Hyman, Ma, Balaguer-Ballester, Durstewitz, & Seamans, 2012; Xu & Südhof, 2013), Rozeske et al. (2015) hypothesized that indirect PL and HPC connectivity contributes to the context specificity of context-shock associations during training in sCFC (see gray lines in

**Figure 2.7**). Prefrontal influence on HPC could be supported via connectivity with the nucleus reunions (NR; part of the VMT) in the absence of direct projections to the dHPC (Fanselow & Dong, 2010; Heroux et al., 2017). The NR is a key ventral midline thalamic structure that has reciprocal connectivity with the mPFC and CA1 region of the dHPC and vHPC, and facilitates communication and oscillatory synchrony between these structures (see Dolleman-van der Weel et al., 2019 for extensive review). Xu & Südhof (2013) used a double-infection strategy in which a cre-dependent adeno-associated virus (AAV) encoding TetTox was injected into NR and a tran-synaptically transported AAV encoding TetTox was injected into mPFC to achieve pathway-specific inhibition of mPFC input into NR during delay fear conditioning. This permanent inhibition prior to training had no effect on cued or contextual fear acquisition but instead caused fear generalization to a non-conditioned context. Increasing NR activity during training by decreasing inhibitory inputs onto NR neurons resulted in less fear generalization, whereas decreasing NR activity resulted in increased fear generalization (Xu & Südhof, 2013). Xu & Südhof (2013) hypothesized that NR neurons control memory generalization by regulating the number of features incorporated into a context representation via connectivity with the HPC. This study provided novel evidence of PFC and NR in controlling the specificity of contextual fear memories encoded during background CFC. In a different study, Ramanathan et al. (2018) showed that pre-training NR inactivation impairs retention but leaves post-shock freezing intact during sCFC (see **Figure 2.8A**). This effect was found to reflect state-dependency as NR inactivation prior to training and testing spared retention test freezing (see **Figure 2.8B**). Interestingly, contextual fear memories formed under NR inactivation were not susceptible to impairments caused

by concurrent NMDAR antagonism in the HPC, which impair fear retention in an otherwise intact rat (see **Figure 2.8C**; Ramanathan, Ressler, Jin, & Maren, 2018). Taken together with Xu & Südhof (2013), these data suggest that NR activity, likely within an mPFC-NR-HPC system, is involved in the formation of precise, hippocampal–dependent context representations or contextual fear memories. Collectively, these and earlier pharmacological studies support and expand Rozeske's (2015) proposed model to emphasize a role of prefrontal-hippocampal circuitry in contextual processes of CFC.

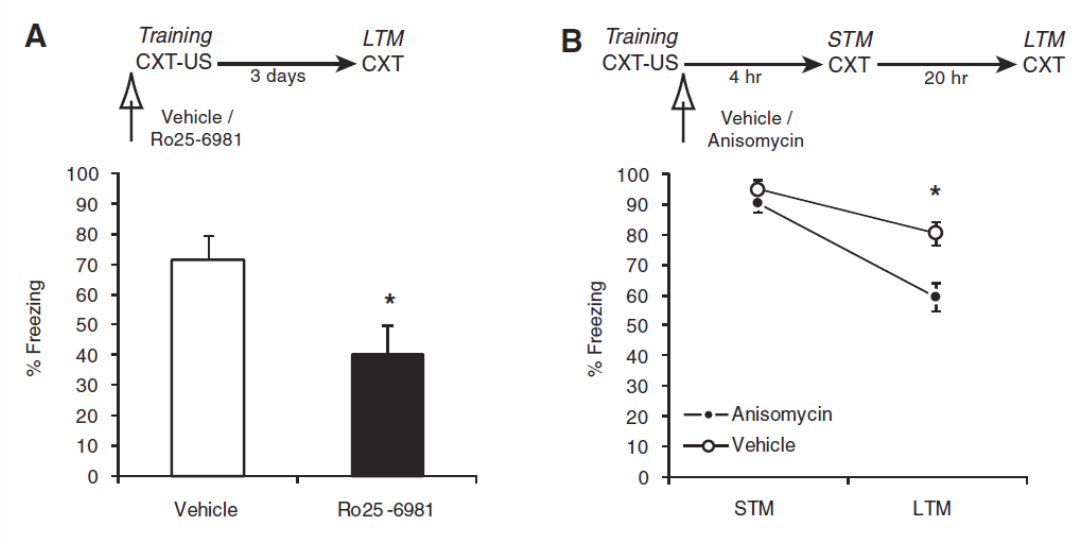


Figure 2.6 Effects of pre-training NMDA-receptor antagonism (via Ro25-6981, A) or post-training protein synthesis inhibition (via anisomycin, B) in the AC during sCFC. **(A)** Pre-training infusion of Ro25-6981 into the AC impairs retention test freezing relative to vehicle infusion. **(B)** Post-training infusion of anisomycin into the AC impairs 24hr but not 4hr retention test freezing. Taken and adapted from Einarsson & Nader, 2011).

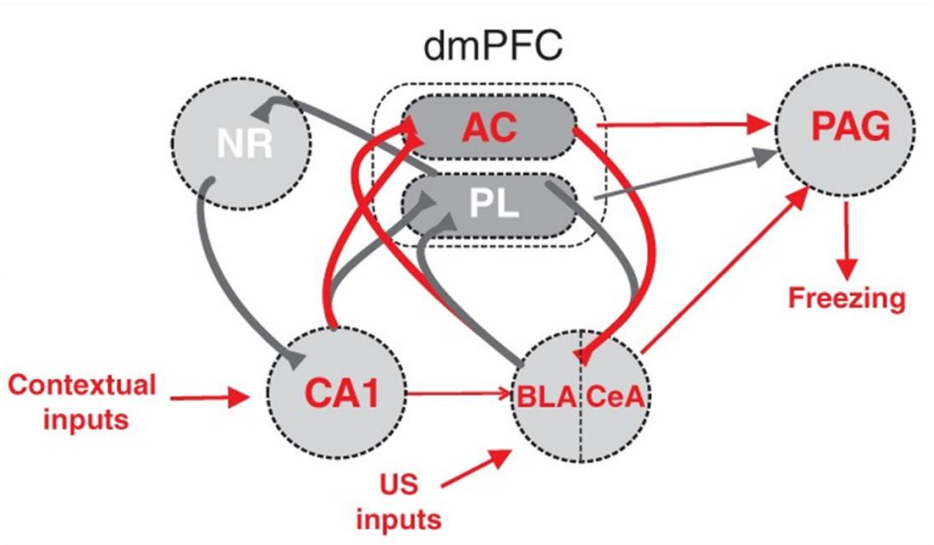


Figure 2.7 Neuronal circuit mediating contextual fear acquisition and retention (red lines indicate proposed circuitry known to be engaged during acquisition, whereas gray lines indicate hypothesized but untested functional connectivity). Contextual fear acquisition is support by contextual inputs into the HPC, which are then sent to the BLA where they converge with US inputs to form a context-shock association. The HPC and BLA also project to the AC, which modulates consolidation of the context-shock association and fear expression via direct PAG and CeA-PAG connectivity. Hypothesized functional connections include HPC relay of contextual information to the PL which, via NR projections, modulates the encoding or consolidation of precise, feature-rich context representations, thus controlling fear generalization during CFC. Taken and adapted from Rozeske et al. (2015).

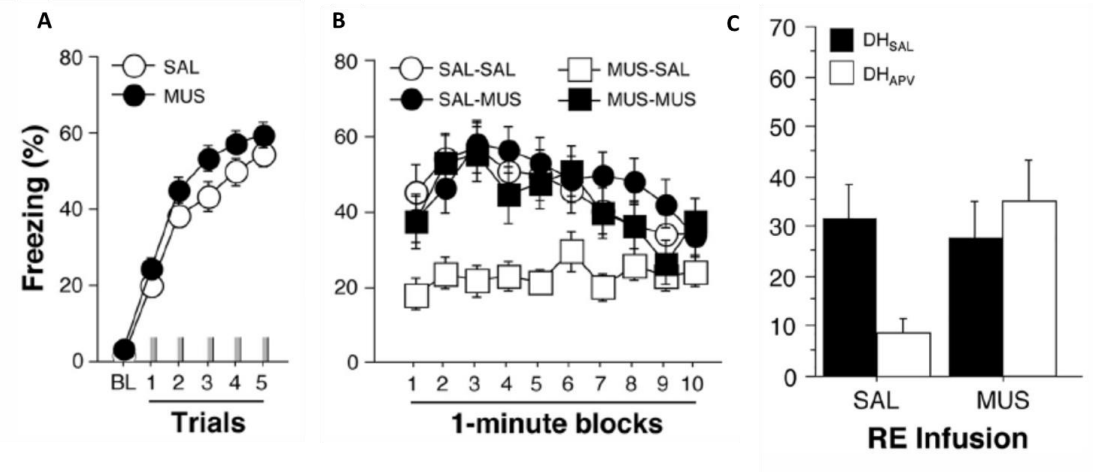


Figure 2.8 Effects of intra-NR infusion of saline or muscimol prior to both training (post-shock freezing; A) and testing (retention freezing; B) during sCFC. **(A-B)** NR inactivation state-dependently impaired retention but not acquisition of sCFC, as MUS-SAL rats froze lower than all other groups during retention. **(C)** Contextual fear memories formed under NR inactivation are insusceptible to concurrent intra-dHPC APV infusion. In the absence of NR inactivation, intra-dHPC APV infusion impairs retention test freezing. Taken and adapted from Ramanathan et al. (2018), who use an alternate acronym (RE) for nucleus reunions.

### 2.1.3 Prefrontal and Hippocampal Dynamics of Contextual Memory in the CPFE

As reviewed in **Chapter 1**, the CPFE is a variant of contextual fear conditioning that separates learning about the context, acquiring a context-shock association, and retrieval/expression of this association (see **Figure 1.1**). Acquisition or consolidation of a context representation requires activity and NMDAR plasticity in both the dHPC and vHPC (Cullen et al., 2017; Matus-Amat, Higgins, Sprunger, Wright-Hardesty, & Rudy, 2007; Schiffino et al., 2011). In addition, inactivation of the whole mPFC prior to any phase of the CPFE abolishes retention test freezing in rats (see **Section 4.2** in **Chapter 4** for full review; Heroux et al., 2017). This inactivation prior to immediate-shock training leaves post-shock freezing intact, indicating that mPFC is not required for retrieving the context representation or associating it with shock. These data suggest that mPFC is necessary for the consolidation of a context representation and/or context-shock association in the CPFE, and that other structure don't compensate for impaired function of mPFC. Taken together with Xu & Südhof (2013) and Ramanathan et al. (2018), these results suggest that mPFC inactivation impairs the CPFE by interfering with mPFC-NR-HPC circuitry recruited for the acquisition or consolidation of a precise, hippocampal-dependent conjunctive context representation. These effects could reflect modulatory influences of mPFC on dHPC (via NR) or vHPC activity, or a reciprocal neural interaction between these two structures (i.e., in which activity in both structures affect each other). These data ultimately led us to establish a hypothesis by which prefrontal-hippocampal interaction support configural learning and memory during context preexposure in the CPFE. Several additional lines of evidence support the formation of this hypothesis. First, both of these structures show robust gene expression in

response to context exposure in CFC, suggesting that they both process contexts (Heroux, Horgan, Rosen, & Stanton, 2019; Heroux et al., 2018; Heroux, Robinson-Drummer, Kawan, Rosen, & Stanton, 2019; Schreiber, Asok, Jablonski, Rosen, & Stanton, 2014; Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014). Rozeske's (2015) model would suggest that this prefrontal activation during context exposure is driven by contextual inputs coming from the HPC, which, via projections back to HPC via NR, would support a hypothesis that PFC and HPC interact cooperatively to produce context learning. Although this cooperative role of HPC is not known, prefrontal neurons respond to context exposure, and activity patterns of these neurons can be location-specific and different across separate spatial contexts (Hyman et al., 2012; Zelikowsky et al., 2014). Second, mPFC underlies sparing of behavioral performance of sCFC after hippocampal damage (Wiltgen et al., 2006; Zelikowsky, Bissiere, & Fanselow, 2012; Zelikowsky et al., 2013). While this may suggest redundancy within the circuit involving parallel processing of contexts by the PFC and HPC during CFC, very little sparing of performance in the CPFE occurs after HPC disruption (Matus-Amat et al., 2004; Robinson-Drummer, Dokovna, Heroux, & Stanton, 2016; Rudy et al., 2002). This indicates that PFC cannot compensate for HPC damage via parallel processing during context exposure in the CPFE, likely because it needs to receive contextual inputs from the HPC (see **Figure 2.7**). Third, additional evidence for a role of this PFC-NR-HPC circuit comes from studies showing pharmacological or optogenetic silencing of NR during sample phases of spatial delayed-non-match-to-position disrupts task performance, neural activity and oscillatory synchrony between the PFC and HPC (Hallock, Wang, & Griffin, 2016; Maisson, Gemzik, & Griffin, 2018). Furthermore, mPFC or NR lesions disrupt

hippocampal representations of space by modulating place field stability and precision (Cholvin, Hok, Giorgi, Chaillan, & Poucet, 2017; Hok, Chah, Save, & Poucet, 2013; Hok, Save, Lenck-Santini, & Poucet, 2005; Kyd & Bilkey, 2003, 2005). Collectively, this research characterizes circuitry that could underlie PFC and HPC involvement in incidental context learning and memory. Accordingly, impairments in conditioning in the CPFE caused by prefrontal inactivation during context exposure could reflect downstream effects on HPC, which in turn projects back to PFC via NR (see Heroux et al., 2017). This hypothesis is further explored in Aim 1 of this dissertation (see Chapter 6).

## 2.2 Molecular Substrates of Contextual Fear Conditioning: Immediate Early Genes

At the core of most biological models of memory lies stabilization and cellular consolidation of various forms of experience-driven synaptic plasticity, mediated through *de novo* gene expression and protein synthesis (Alberini, 2009; Kandel, Dudai, & Mayford, 2014). Long-term potentiation (LTP) is an enhancement of synaptic strength and efficacy resulting from strong synaptic stimulation primarily driven by sensory and behavioral experiences (Minatohara, Akiyoshi, & Okuno, 2016). Molecular mechanisms of LTP induction include the depolarization of postsynaptic cells via glutamatergic AMPA- and NMDA-receptor activation, resulting in intracellular $Ca^{2+}$ influx and AMPA-receptor trafficking into the postsynaptic membrane. This $Ca^{2+}$ influx underlies the stabilization of LTP via activation of multiple intracellular signaling cascades leading to *de novo* transcription and translation of plasticity-associated genes (Kandel et al., 2014). Immediate early genes represent a subset of these genes in which transcription is rapidly and transiently

induced by this neural activity and do not require prior protein synthesis (Mukherjee et al., 2018). The IEGs *c-Fos*, early-growth-response-gene-1 (*Egr-1*), and neuronal PAS domain protein 4 (*Npas4*) are transcription factors that regulate the expression of other downstream late-response effector genes that support long-term memory, whereas activity-regulated cytoskeleton-associated protein (*Arc*) encodes a protein that directly regulates dendritic synaptic plasticity (Gallo, Katche, Morici, Medina, & Weisstaub, 2018; Minatohara et al., 2016). In the current dissertation, we examine expression of these IEGs as correlational, regional markers of neural activity and plasticity during discrete behavioral processes of the CPFE in normally-reared and neonatal-alcohol-exposed adolescent rats (see Chapters 6-8). Although their role in supporting memory is likely universal, I include a basic overview of research probing the link between these IEGs and learning and memory in CFC and similar behavioral tasks in rodents in the following sections.

### 2.2.1 Immediate Early Genes: *c-Fos*

A component of the transcription factor AP-1, the IEG *c-Fos* is induced by intracellular $Ca^{2+}$ and CREB signaling and has historically been used as a general marker of neural activity seen within minutes of behavioral experience (Alberini, 2009; J. Morgan, 1991). Early studies demonstrated a correlational link between *c-Fos* expression in limbic structures and behavioral performance of sCFC, signaled fear conditioning, spatial memory, and conditional discrimination tasks in mice and rats (Amin, Pearce, Brown, & Aggleton, 2006; Guzowski, Setlow, Wagner, & McGaugh, 2001; Hess, Lynch, & Gall, 1995; Huff et al., 2006; Nagahara & Handa, 1995; Radulovic, Kammermeier, & Spiess, 1998; Rosen, Fanselow, Young, Sitcoske, & Maren, 1998; Vann, Brown, & Aggleton, 2000; Vann, Brown, Erichsen, & Aggleton,

2000). Mutant or transgenic *c-Fos* knockout mice have impaired hippocampal NMDAR-mediated LTP induction and show deficits in retention of CFC and place learning in Morris water maze (MWM; Fleischmann et al., 2003; Paylor, Johnson, Papaioannou, Spiegelman, & Wehner, 1994). Pharmacological disruption of *c-Fos* expression via intra-hippocampal infusion of *c-Fos* antisense oligonucleotides (ASO) impairs retention of MWM, and object location recognition, and visual conditional discrimination tasks in rats (Grimm et al., 1997; Guzowski, 2002; Guzowski & McGaugh, 2002; Kemp, Tischmeyer, & Manahan-Vaughan, 2013). In inhibitory avoidance tasks, post-training ASO infusion into the hippocampus or retrosplenial cortex disrupts the retention and persistence of long-term memory (Katche et al., 2013; Katche, Goldin, Gonzalez, Bekinschtein, & Medina, 2012; Katche & Medina, 2017; Slipczuk et al., 2009). These studies suggest that *c-Fos* expression induced by neural activity during behavioral experience plays a role in subsequent cellular consolidation of memory. This notion is strongly supported by recent research using optogenetic excitation or inhibition of hippocampal, amygdalar, prefrontal, or retrosplenial neurons expressing *c-Fos* during acquisition in cued and contextual fear conditioning (Cai et al., 2016; Cowansage et al., 2014; DeNardo et al., 2019; Liu et al., 2012; Matsuo, 2015; Miyashita, Kikuchi, Horiuchi, & Saitoe, 2018; Ramirez et al., 2013; Rashid et al., 2016; Tanaka et al., 2014). For example, as noted above (**Figure 2.5**), optogenetic activation of hippocampal neurons expressing *c-Fos* during CFC acquisition results in expression of fear regardless of testing context (Liu et al., 2012). Taken together, these studies suggest a functional role of c-Fos-expressing neurons in the long-term memory trace.

### 2.2.2 Immediate Early Genes: *Arc*

The IEG *Arc* codes for a protein that directly regulates dendritic synaptic plasticity, and supports consolidation and persistence of long-term memory that depends on limbic structures, particularly the hippocampus (Korb & Finkbeiner, 2011). Neural *Arc* induction serves several behaviorally-relevant functions (see Minatohara et al., 2016). First, *Arc* regulates synaptic scaling by local transport into dendritic spines where it facilitates AMPAR endocytosis in weak or silent synapses, and augments AMPAR exocytosis in activated synapses. Second, previously translated *Arc* protein can augment LTP induced by weak learning that would otherwise not be consolidated into long-term memory. Third, untranslated *Arc* transcripts can be locally transported to dendritic compartments where they can subsequently effect LTP and long-term depression (LTD) processes induced by sensory or behavioral experience. Finally, *Arc* stabilizes dendritic F-Actin expression induced by activity, which is involved in dendritic remodeling that supports consolidation of LTP (Fukazawa et al., 2003; Messaoudi et al., 2007). Interestingly, unlike *c-Fos* or *Egr-1*, *Arc* expression has been suggested to have a selective or preferential role in spatial learning and memory engaging the hippocampus (Guzowski et al., 2001; Lonergan, Gafford, Jarome, & Helmstetter, 2010). Context exposure during CFC induces *Arc* expression in the PFC and HPC regardless of foot-shock presentation (Heroux et al., 2018; Huff et al., 2006; Lonergan et al., 2010; Pevzner & Guzowski, 2015; Zelikowsky et al., 2014). One study examined the time-course of *Arc* expression in the HPC in response to varying amounts of context exposure occurring before foot-shock in rats (Pevzner & Guzowski, 2015). Rats given 30s but not 3s of context exposure showed maximal CA1 *Arc* expression (identical to the 300s group), which corresponds to the amount of time required to form a context representation in CFC (Fanselow, 1990; Pevzner &

Guzowski, 2015). Transgenic *Arc* knockout mice show deficits in retention of MWM, CFC, conditioned taste aversion, and novel object recognition, concurrent with disruptions in spine morphology and late-phase LTP consolidation (Peebles et al., 2010; Plath et al., 2006). In addition, local hippocampal or amygdalar *Arc* ASO infusion during training disrupts these tasks in rodents (Czerniawski et al., 2011; Guzowski et al., 2000; Nakayama et al., 2016, 2015). Taken together, these studies implicate a role of *Arc* in regulating synaptic plasticity and memory consolidation engaging hippocampal circuitry.

### 2.2.3 Immediate Early Genes: *Egr-1*

The IEG *Egr-1* codes for a transcription factor which regulates the expression of other late-phase plasticity-related proteins supporting memory consolidation across a wide range of behavioral tasks (Gallo et al., 2018). *Egr-1* is induced in the HPC, PFC, and AMY during un-signaled and signaled CFC, inhibitory avoidance, and MWM tasks in rodents (Donley & Rosen, 2017; Guzowski et al., 2001; Hall, Thomas, & Everitt, 2001; Malkani & Rosen, 2000; Nikolaev, Kaminska, Tischmeyer, Matthies, & Kaczmarek, 1992; Rosen et al., 1998; Stern, Gazarini, Vanvossen, Hames, & Bertoglio, 2014). Our lab has shown that both context preexposure and immediate-shock training induces expression of *Egr-1* in the mPFC, dHPC, and LA (Asok, Schreiber, Jablonski, Rosen, & Stanton, 2013; Heroux et al., 2018; Schreiber et al., 2014). Interestingly, the only region in which learning-related expression above non-associative controls is seen is the mPFC, but this expression is not required for CPFE performance (see **Chapter 4** for review; Robinson-Drummer, Chakraborty, Heroux, Rosen, & Stanton, 2018). Transgenic *Egr-1* knockout or mutant mice exhibit deficits in long-term but not short-term memory in CFC, MWM, object-in-place recognition,

and conditioned taste aversion, concurrent with impaired late-phase LTP consolidation (Besnard, Caboche, & Laroche, 2013; Bozon, Davis, & Laroche, 2003; Davis et al., 2010; Gallo et al., 2018; S. Han et al., 2014; Jones, Errington, & French, 2001). Depletion of *Egr-1* expression via local ASO or NMDAR-antagonist infusion into the HPC, PFC, or AMY disrupts long-term memory in many of these behavioral tasks (Katche et al., 2012; J. L. Lee, 2008, 2010; Maddox, Monsey, & Schafe, 2011; Malkani & Rosen, 2001; Malkani, Wallace, Donley, & Rosen, 2004). One common finding is that regional *Egr-1* expression may have a functional role in memory reconsolidation or updating (J. L. Lee, 2008, 2010; Maddox et al., 2011; Stern et al., 2014). Taken together, these latter studies suggest a role of *Egr-1* expression in late-phase consolidation and updating of aversive memory.

### 2.2.4 Immediate Early Genes: *Npas4*

The IEG *Npas4* codes for a neuron-specific transcription factor that is rapidly and exclusively induced by intracellular $Ca^{2+}$ signaling and neural activity, compared to *c-Fos*, *Arc*, and *Egr-1*, which are expressed in other cells and can be induced by stimuli such as growth factors, neurotrophins, or kinase signaling (Lin et al., 2008; Sun & Lin, 2016). *Npas4* is thought to modulate plasticity in both excitatory and inhibitory synapses by regulating secondary waves of neurotrophic factor and IEG expression after neuronal activation (Bloodgood, Sharma, Browne, Trepman, & Greenberg, 2013; Sun & Lin, 2016). Unlike other IEGs, there are very few studies measuring or manipulating *Npas4* expression during behavior in rodents. *Npas4* is rapidly expressed in the hippocampus and lateral amygdala during cued and contextual fear conditioning, respectively (Ploski, Monsey, Nguyen, DiLeone, & Schafe, 2011; Ploski, Park, Ping, Monsey, & Schafe, 2010; Ramamoorthi et al., 2011; Weng et al.,

2018). In CFC, there's no difference in hippocampal *Npas4* expression between rats receiving context exposure and context-shock pairing, with both groups showing higher expression than an immediate-shock control group (Ramamoorthi et al., 2011). This suggests that hippocampal *Npas4* expression is specific to incidental contextual learning processes of CFC. Transgenic *Npas4* knockout mice show selective deficits in contextual but not cued fear conditioning (Ramamoorthi et al., 2011). Interestingly, depletion of CA3 but not CA1 *Npas4* expression impairs CFC, indicating a role for sub-region-specific expression in behavioral performance (Ramamoorthi et al., 2011; Weng et al., 2018). Disruption of *Npas4* expression via AAV-mediated gene delivery of micro RNAs (for RNA interference) into the amygdala disrupts the consolidation of new and reactivated cued fear memory (Ploski et al., 2011). Taken together, these studies demonstrate a role of hippocampal and amygdalar *Npas4* expression in cued and contextual fear memory. This dissertation adds to this literature by characterizing *Npas4* expression in extended prefrontal-hippocampal circuitry (i.e., in mPFC, dHPC, vHPC, and VMT) during context exposure in adolescent rats (see **Chapter 6**).

## 2.3 Conclusion

This chapter summarized key studies highlighting the regional neurobiological and molecular substrates of Pavlovian CFC. These studies demonstrate involvement of the HPC, PFC, and AMY in processes of CFC. One difficulty with interpretation of previous CFC research is the inability to fully dissociate mechanisms of context vs. contextual fear memory, as these two processes occur within the same training session in these tasks. This weakness is addressed by CPFE studies because these two processes are temporally dissociated. Despite this, the role of HPC and PFC circuitry in supporting context learning that is incidental vs. reinforcement-driven in CFC is

poorly characterized. Accordingly, this dissertation examines hippocampal and prefrontal mechanisms of incidental context learning and memory in the CPFE in normally-reared and neonatal-alcohol-exposed adolescent rats (see **Chapters 6 and 7**). Taken together with recent research, these studies suggest a key role of prefrontal circuitry in contextual processes of Pavlovian CFC (Gilmartin et al., 2014; Giustino & Maren, 2015; Rozeske et al., 2015).

# Chapter 3

# ANIMAL MODELS OF FETAL ALCOHOL SPECTRUM DISORDER

The preceding chapter described the neurobiology of conditioning in rats, focusing on the involvement of the HPC and PFC in supporting context and contextual fear memory. The present chapter examines the neurobiological and behavioral consequences of developmental alcohol exposure in rodent models of FASDs. The discussion focuses on hippocampal, prefrontal, and cholinergic mechanisms of impaired cognition following third-trimester equivalent alcohol exposure in rats. This research framework is briefly discussed in relation to previous work from our lab and the experiments in this dissertation.

## 3.1 Neurocognitive Consequences of Gestational Alcohol Exposure in Developing Humans

Neurodevelopmental and cognitive impairment resulting from gestational alcohol exposure in humans is a leading preventable cause of severe intellectual disability in the United States and abroad (May et al., 2009; Murawski et al., 2015; Rasmussen, Andrew, Zwaigenbaum, & Tough, 2008). Recent conservative estimates of FASD prevalence place it as high as 5% in diverse US communities, which underscores it as a serious and unanswered societal problem (May et al., 2018). Fetal alcohol spectrum disorders describe an array of somatic, neural, physiological, and cognitive impairments resulting from the teratogenic effects of alcohol on the developing central nervous system. Gestational exposure in humans via maternal consumption during pregnancy decreases cortical thickness and gyrification

(Hendrickson et al., 2017; Yang et al., 2012), limbic gray matter (Donald et al., 2016), brain volume (Rajaprakash, Chakravarty, Lerch, & Rovet, 2014), and neural activity (Norman et al., 2009; Panczakiewicz et al., 2016; Suttie et al., 2018; Wozniak et al., 2016). While the whole brain is affected, key structures particularly vulnerable to the teratogenic effects of alcohol in humans include the cerebellum, hippocampus, striatum, and frontal cortex (Lebel, Roussotte, & Sowell, 2011; Moore, Migliorini, Infante, & Riley, 2014; Spottiswoode et al., 2011; Willoughby, Sheard, Nash, & Rovet, 2008). Hippocampal insult may underlie cognitive deficits resulting from gestational alcohol exposure in humans (Kodituwakku, 2009). For example, prenatally exposed children show performance deficits in probe trials on spatial but not cued MWM (D. A. Hamilton, Kodituwakku, Sutherland, & Savage, 2003) and in learning the spatial arrangement of objects and recalling them after a delay (Uecker & Nadel, 1996; Willoughby et al., 2008). Taken together, these studies suggest that alcohol impairs hippocampal development and function.

In addition to cognition involving the hippocampus, FASDs impair executive function and working memory, which depends on the prefrontal cortex (Marquardt & Brigman, 2016; Nunez, Roussotte, & Sowell, 2011). For example, alcohol-exposed children show abnormal frontal lobe activity during incorrect *n-back* task performance (Malisza et al., 2005). More specifically, these children had fewer correct responses, less responding, and longer latencies for response choice when compared to healthy children. In addition, children with FASD showed greater activity in the frontal cortex that didn't change with task demands, in contrast to a positive correlation between task difficulty and frontal lobe activity in healthy children (Malisza et al., 2005). Another example can be seen with deficits in response inhibition in the "go/no-go" task in

children with FASD, which depends on intact prefrontal function (Fryer et al., 2007). While human research has demonstrated robust alcohol-induced dysfunction in the frontal cortex and associated behaviors, examination of prefrontal mechanisms of impaired cognition in animal model research is lacking. This central issue is a focus of this dissertation.

### 3.2 Animal Models of Fetal Alcohol Spectrum Disorders

Animal models of FASD have been instrumental in isolating the neural and behavioral disruptions caused by developmental alcohol exposure because of the ability to manipulate multiple factors such as developmental window of exposure, pattern of administration, and dosage across development (Patten et al., 2014; Schneider, Moore, & Adkins, 2011). In rat models, the most common methods of administration include maternal consumption of alcohol throughout pregnancy, vapor inhalation, intraperitoneal or subcutaneous injection, and intragastric intubation of neonates. The effects of alcohol on the developing nervous system depend both on the route and timing of alcohol administration (Klintsova, Hamilton, & Boschen, 2013; Lebedeva et al., 2017). In the rat, early and late prenatal exposure to alcohol roughly corresponds to the first and second trimester of human pregnancy, respectively. In humans, the brain growth spurt occurs during the third trimester of pregnancy whereas this event occurs during the early neonatal period in the rat, i.e., approximately PD-PD10 **(**Klintsova, Hamilton, & Boschen, 2013). Therefore, rodent models targeting this sensitive period of brain development use direct alcohol administration to neonatal rat pups rather than maternal consumption (Murawski et al., 2015; Patten et al., 2014). In general, exposure window determines which brain regions are targeted whereas pattern and route of exposure modulate severity of injury via effects of peak BACs

and length of alcohol withdrawal. For example, binge-like exposure via intragastric intubation in rodent pups produces a high peak BAC followed by protracted return to normal levels (Patten et al., 2014; Petrelli, Weinberg, & Hicks, 2018). This period of withdrawal causes glutamatergic excitotoxicity resulting from alcohol's effects as an NMDAR antagonist and GABA receptor agonist in the developing nervous system (Ikonomidou, Price, Stefovska, & Ho, 2000). Every experiment in this dissertation uses intragastric intubation in neonatal rat pups to model third-trimester equivalent exposure.

### 3.3 Hippocampal Insult after Neonatal Alcohol Exposure in Rats

Neonatal alcohol exposure in rats captures many key aspects of disrupted brain and behavior seen in human FASD (Murawski et al., 2015; Patten et al., 2014). While disrupted cerebellar functioning is perhaps the most thoroughly established parallel between the human condition and animal models of FASD (Brown, Goodlett, & Stanton, 2007; Cheng et al., 2015; Jacobson et al., 2018, 2011, 2008; Stanton & Goodlett, 1998), impaired hippocampal function has dominated animal research for a longer period (Berman & Hannigan, 2000). In rats, alcohol exposure during PD4-10, PD4-9, or PD7-9 decreases hippocampal CA1 pyramidal cell counts, while CA3 and DG neurons are less sensitive to exposure (Bonthius & West, 1990; Livy, Miller, Maier, & West, 2003; Marino et al., 2004; Murawski et al., 2012). Sensitivity of CA1, CA3, and DG neurons to alcohol insult is greatest during this third-trimester equivalent period in the rat (Livy et al., 2003). Indeed, alcohol-induced hippocampal cell loss after PD4-9 exposure is equivalent to reductions seen after combined E1-20 and PD4-9 exposure. In addition to cell loss, neonatal alcohol exposure increases hippocampal neuroinflammation, cytokine production (e.g., *IL1b* and *TNF*), DNA

methyltransferase activity, and global DNA methylation in rats (Goodfellow, Shin, & Lindquist, 2018; Otero, Thomas, Saski, Xia, & Kelly, 2012; Perkins, Lehmann, Lawrence, & Kelly, 2013). Reducing this neuroinflammation via concurrent ibuprofen administration during alcohol dosing ameliorates increased cytokine production and deficits in trace fear conditioning in adolescent rats (Goodfellow et al., 2018). Interestingly, administration of the acetylcholinesterase inhibitor, physostigmine, prior to contextual or trace fear conditioning also rescues alcohol-induced behavioral impairments (Dokovna et al., 2013; Hunt & Barnet, 2015). These findings suggest that developmental alcohol exposure causes lasting disruptions in hippocampal neuroimmune and cholinergic function that interfere with behavior, as acetylcholinesterase inhibitors also have anti-inflammatory functions (Kalb et al., 2013; Pope, Karanth, & Liu, 2005). Finally, neonatal alcohol exposure significantly alters hippocampal synaptic plasticity in the rat. Alcohol treatment alters MAPK/ERK signaling and expression of the GluN2B NMDAR subunit, PSD-95, and muscarinic $M_1$ and $M_{2/4}$ receptors in the hippocampus (DuPont, Coppola, Kaercher, & Lindquist, 2014; Goodfellow, Abdulla, & Lindquist, 2016; Monk, Leslie, & Thomas, 2012). In addition, acute or chronic alcohol exposure abolishes the induction and maintenance of LTP and AMPAR and NMDAR-mediated excitatory postsynaptic potentials (EPSPs) in the CA1 *ex vivo* (Puglia & Valenzuela, 2010b, 2010a). Taken together, these studies demonstrate the vulnerability of glutamatergic and cholinergic function in the hippocampus to developmental alcohol exposure. In summary, the first ten days of life in the rat represents a critical period for alcohol-induced disruption of hippocampal neuroanatomy and function.

### 3.4 Prefrontal Insult after Neonatal Alcohol Exposure in Rats

As in the human condition of FASD (Section 3.1), developmental alcohol exposure also alters medial prefrontal neuroanatomy and function in rodents. However, examination of this issue in rodent models is relatively limited. In contrast to the lasting cell loss seen in the hippocampus, neonatal alcohol exposure has no lasting effect on prefrontal pyramidal cell number but instead alters synaptic plasticity, dendritic complexity, gene expression, and epigenetic markers (Boschen, Keller, Roth, & Klintsova, 2018; Granato, Di Rocco, Zumbo, Toesca, & Giannetti, 2003; Granato, Palmer, De Giorgio, Tavian, & Larkum, 2012; G. F. Hamilton, Whitcher, & Klintsova, 2010; Otero et al., 2012). In contrast to pyramidal cell counts, neonatal exposure does decrease parvalbumin interneurons in the AC but not PL or IL in adult rats (G. F. Hamilton, Hernandez, Krebs, Bucko, & Rhodes, 2017). Neonatal alcohol exposure also decreases dendritic complexity and density that may alter circuit organization and behavior in rats (G. F. Hamilton et al., 2010; Lawrence, Otero, & Kelly, 2012; Whitcher & Klintsova, 2008). Exposure across prenatal and neonatal development decreases apical and basilar spine density and complexity in layer II/III pyramidal cells in the mPFC of adult rats (Lawrence et al., 2012). Exposure limited to the third-trimester equivalent causes reduced apical dendrite density and basilar dendrite complexity in prefrontal pyramidal neurons also in adolescent rats (Granato et al., 2003; G. F. Hamilton et al., 2010; Whitcher & Klintsova, 2008). Neonatal exposure also alters voltage-gated $Ca^{2+}$ channel activity while decreasing the amount and duration of dendritic spiking in layer V pyramidal neurons in the PFC (Granato et al., 2012). Taken together with increased global DNA methylation (Boschen et al., 2018; Otero et al., 2012), these physiological changes likely alter activity and plasticity-associated gene expression supporting learning and memory.

While studies of neonatal exposure are limited, *prenatal* alcohol exposure in rats alters experience-dependent gene expression in the medial prefrontal cortex. For example, ethanol exposure throughout gestation decreases expression of the IEGs *Arc* and *c-Fos* in the PL in adult rats during wrestling and social interaction tasks (D. A. Hamilton, Akers, et al., 2010; D. A. Hamilton, Candelaria-Cook, et al., 2010). A narrower second-trimester equivalent exposure disrupts the expression of the transcription factors *c-Fos* and *jun-B* in the PL and ACC during T-maze alternation (Nagahara & Handa, 1995). Finally, late gestational exposure also decreases *c-Fos* protein expression in the IL during open field exploration in adolescent rats (Fabio et al., 2013). More research is needed to establish a link between prefrontal targeting and cognitive deficits resulting from *neonatal* ethanol exposure in rats. **Chapters 7 and 8** in this dissertation examine this issue by measuring activity and plasticity-associated gene expression in the prefrontal cortex of rats receiving neonatal alcohol exposure.

### 3.5 Cholinergic Insult after Neonatal Alcohol Exposure in Rats

Animal model research suggests that alcohol exposure disrupts acute and chronic cholinergic muscarinic-receptor cell signaling involved in neuronal and microglial proliferation, synaptic plasticity, and behavioral performance on tasks involving hippocampal and prefrontal cholinergic activity (Costa, Giordano, & Guizzetti, 2013; Monk et al., 2012; Wilhelm & Guizzetti, 2015). In neonatal rats, alcohol decreases the bioavailability of the acetylcholine precursor choline and inhibits glial muscarinic-receptor cell signaling important for neuritogenesis in proliferating hippocampal pyramidal neurons (Costa et al., 2013; Giordano, Guizzetti, Dao, Mattison, & Costa, 2011; Goeke, Roberts, Hashimoto, Finn, & Guizzetti, 2018; Guizzetti, Moore, Giordano, VanDeMark, & Costa, 2010; Guizzetti, Moore,

VanDeMark, Giordano, & Costa, 2011). Importantly, alcohol-induced disruptions in cholinergic signaling are not limited to the period of alcohol exposure (Monk et al., 2012). Decrease in hippocampal $M_1$ muscarinic receptors and a significant increase in $M_{2/4}$ receptors are seen in adolescent rats. This change in hippocampal muscarinic receptor composition likely interferes with synaptic plasticity via increased activation of $M_{2/4}$ receptors, which downregulate cyclic AMP activity important for induction of transcription factors and calcium channel activity (Migeon & Nathansons, 1994; Monk et al., 2012). Whether this lasting interference with muscarinic receptor composition and signaling is seen in other brain regions such as the prefrontal cortex is unknown and remains a fruitful direction for future animal model research.

A growing body of work suggests that increasing bioavailability of choline and thus cholinergic signaling across development has potential as a therapeutic intervention for FASD in rodent models. Alcohol-induced impairment of MWM, fear conditioning, spatial discrimination, working memory, and motor behavior are attenuated by developmental choline supplementation in rats (Hunt & Barnet, 2015; Idrus, Breit, & Thomas, 2017; Ryan, Williams, & Thomas, 2008; Thomas, Abou, & Dominguez, 2009; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007; Thomas, Garrison, & O'Neill, 2004; Thomas, Idrus, Monk, & Dominguez, 2010; Wagner & Hunt, 2006). In addition to these behavioral effects, developmental choline administration reverses increased global DNA methylation in the HPC and PFC, which in the absence of choline causes a restrictive state for activity-driven gene expression (Otero et al., 2012; Perkins et al., 2013). This choline administration also attenuates alcohol-induced increases in $M_{2/4}$ muscarinic receptors in the HPC, which likely results in decreased inhibition of cyclic AMP activity (Monk et al., 2012).

While more research is needed, these benefits of choline supplementation during gestation are also promising in the human condition (Jacobson et al., 2018; Wozniak et al., 2015, 2013). Clearly, the specific long-term therapeutic mechanisms of choline supplementation on neurobiological and behavioral outcome measures in humans and animal models demands further investigation. Moreover, the effects of enhancing cholinergic system function acutely later in life on cognition is not well characterized.

### 3.6 Behavioral Consequences of Neonatal Alcohol Exposure in Rats

Animal model research examining third-trimester equivalent alcohol exposure (e.g., from PD7-9 or PD4-9) in rats has focused on establishing a link between altered hippocampal development and behavioral performance on traditionally "hippocampal-dependent" behavioral tasks. Neonatal alcohol exposure has the most disruptive effects on "hippocampus-dependent" tasks that also depend on PFC. For example, neonatal alcohol impairs later spatial learning and memory in place but not cued MWM procedures, especially during probe trials with long delays (Girard, Xing, Ward, & Wainwright, 2000; Goodlett & Johnson, 1997; Goodlett & Peterson, 1995; Johnson & Goodlett, 2002; Marino et al., 2004; Thomas et al., 2007; Thomas, Sather, & Whinery, 2008). However, lesions or pharmacological disruption of the mPFC in rats also interfere with allocentric spatial learning in the MWM (Fantie & Kolb, 1990; Kolb, Buhrmann, McDonald, & Sutherland, 1994; Kolb & Cioe, 1996; Leon, Bruno, Allard, Nader, & Cuello, 2010; McDonald, King, Foong, Rizos, & Hong, 2008; Sang Jo et al., 2007). Consistent with potential prefrontal mechanisms of MWM disruption, rescuing hippocampal CA1 cell loss by developmental Vitamin E supplementation (Marino et al., 2004; see **Figure 3.1**) does not rescue probe trial performance in rats after PD7-9 exposure (Marino et al., 2004). Alcohol-exposed rats also show deficits on

delay and trace eyeblink conditioning (Brown, Calizo, & Stanton, 2008; Brown et al., 2007; Lindquist, Sokoloff, Milner, & Steinmetz, 2013; Murawski, Jablonski, Brown, & Stanton, 2013) as well as trace and contextual fear conditioning (Dokovna et al., 2013; DuPont et al., 2014; Goodfellow et al., 2016, 2018; Hunt & Barnet, 2015; Hunt, Jacobson, & Torok, 2009; Jablonski et al., 2018; Jablonski & Stanton, 2014; Murawski et al., 2012; Murawski & Stanton, 2010, 2011; Wagner & Hunt, 2006). Trace eyeblink, trace fear, and background contextual fear conditioning are also disrupted by *prefrontal* lesions, pharmacological inactivation, or local glutamatergic receptor antagonism in rodents (Beeman et al., 2013; Gilmartin, Miyawaki, et al., 2013; Kalmbach, Ohyama, Kreider, Riusech, & Mauk, 2009; Siegel et al., 2015; Stern et al., 2014; Takehara-Nishiuchi, Kawahara, & Kirino, 2005). Indeed, acquisition and consolidation of a long-term trace fear conditioning memory generally depends on activity and NMDA-receptor plasticity in the mPFC, dHPC, and vHPC in rats (**Chapter 2;** Beeman et al., 2013; Chowdhury, Quinn, & Fanselow, 2005; Gilmartin & Helmstetter, 2010; Gilmartin et al., 2012; Gilmartin, Kwapis, et al., 2013). Taken together, these studies demonstrate that alcohol-induced neurobehavioral deficits that traditionally have been solely attributed to impaired hippocampal development could just as likely be attributed to impaired PFC function.

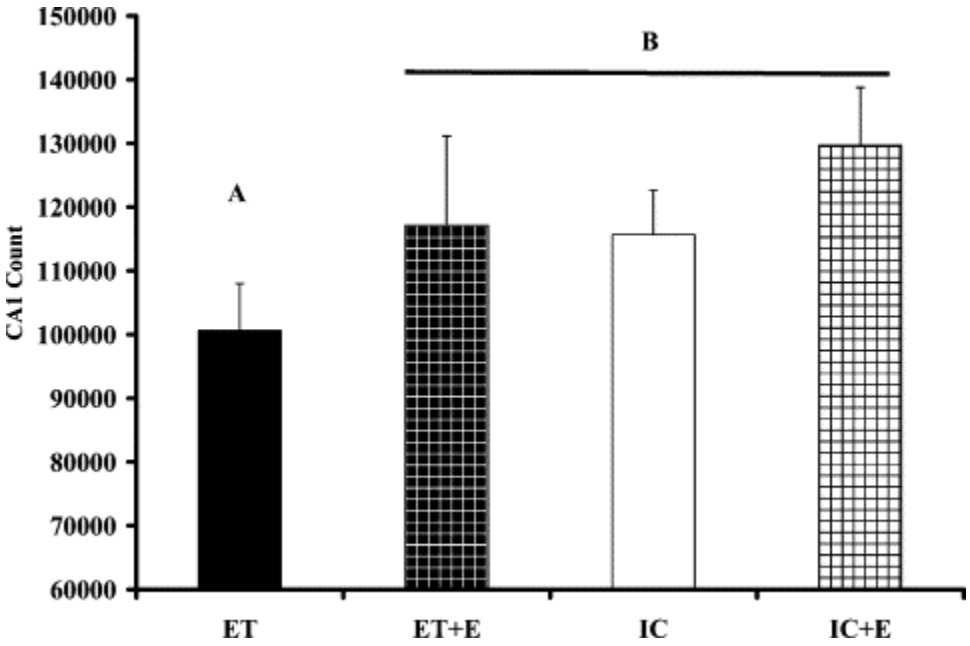


Figure 3.1 Unbiased stereology data for CA1 region of the hippocampus. CA1 pyramidal cell number estimates by unbiased stereology. Unilateral cell number estimates are reported. Groups with different letters were significantly different. Error bars represent S.E.M. ET= ethanol-exposed rats, E = vitamin E treatment, IC = intubated control. Developmental vitamin E treatment eliminated differences in CA1 pyramidal cell counts between the ET and IC groups. Taken from Marino et al., 2004.

### 3.7 Impairment of the CPFE by Neonatal Alcohol Exposure in Rats

Our lab has shown that the CPFE is particularly sensitive to disrupted HPC and PFC function resulting from third-trimester equivalent alcohol exposure in rats. The CPFE requires both activity and cholinergic muscarinic-receptor cell signaling in the dHPC and mPFC during all three phases (Heroux et al., 2017; Matus-Amat et al., 2004; Robinson-Drummer et al., 2016, 2017). Our lab has consistently reported robust impairments in retention test freezing during the CPFE in adolescent and adult rats after neonatal alcohol exposure from PD7-9 or PD4-9 (G. F. Hamilton et al., 2011; Jablonski et al., 2018; Jablonski & Stanton, 2014; Murawski et al., 2012; Murawski & Stanton, 2010, 2011). Moreover, impairment of performance scales with alcohol dose, with a significant negative correlation of BACs during exposure and test freezing (Murawski & Stanton, 2011). The specific effects of neonatal alcohol dosing on learning and memory processes in the CPFE depend largely on the dosing window. Our lab has examined behavioral performance in adolescent rats after neonatal alcohol exposure from PD7-9, PD4-6, and PD4-9 (Murawski & Stanton, 2011). Unlike after PD4-9 or PD7-9, PD4-6 alcohol exposure has no effect on retention test freezing in the CPFE (Murawski & Stanton, 2011). The notion that exposure over PD7-9 window alone is sufficient to capture alcohol-induced behavioral deficits is supported by previous research showing that PD7-9 and PD4-9, but not PD4-6, alcohol exposure impairs performance in the MWM (Goodlett & Johnson, 1997; Goodlett & Peterson, 1995). Further examination of the PD7-9 window has shown that exposed rats have intact post-shock but impaired retention test freezing in the CPFE (Jablonski & Stanton, 2014). These data indicate that PD7-9 alcohol-exposed rats are able to form a context-shock association but consolidation of this association is impaired. This notion is supported by the finding that alcohol-exposed rats have reduced prefrontal

expression of the IEG *egr-1* after preexposure and training (Jablonski et al., 2018). Interestingly, while these data suggest that PD4-6 exposure is not relevant to behavioral disruption of the CPFE, PD4-9 alcohol exposure results in knockdown of hippocampal *c-Fos* protein expression and CA1 pyramidal cell loss on the preexposure day (Murawski et al., 2012; see **Figure 3.2**). In addition, reducing the interval between context preexposure and training to 2hr instead of 24hr rescues retention test freezing in alcohol-exposed animals, further suggesting additional impairment of preexposure day processes after PD4-9 exposure (Goodfellow & Lindquist, 2014). The effect of this exposure on prefrontal activity during context learning and post-shock freezing in the CPFE is not known. Given the early timing of neonatal prefrontal development (Ferguson & Gao, 2014; van Eden & Uylings, 1985; Z. wei Zhang, 2004), PD4-9 alcohol exposure may alter prefrontal function important for behavior across the lifespan. Finally, our lab has shown that administration of the acetylcholinesterase inhibitor physostigmine (PHY) prior to all three phases of the CPFE rescues retention test freezing in PD7-9 ethanol-exposed rats, indicating a role for disrupted cholinergic signaling in the alcohol-induced behavioral deficit (Dokovna et al., 2013; see **Figure 3.3**). The specific memory processes of the CPFE as well as regional neural and cholinergic function that is disrupted by neonatal alcohol exposure are not well characterized. These questions are explored further in **Chapters 7 and 8** of this dissertation.

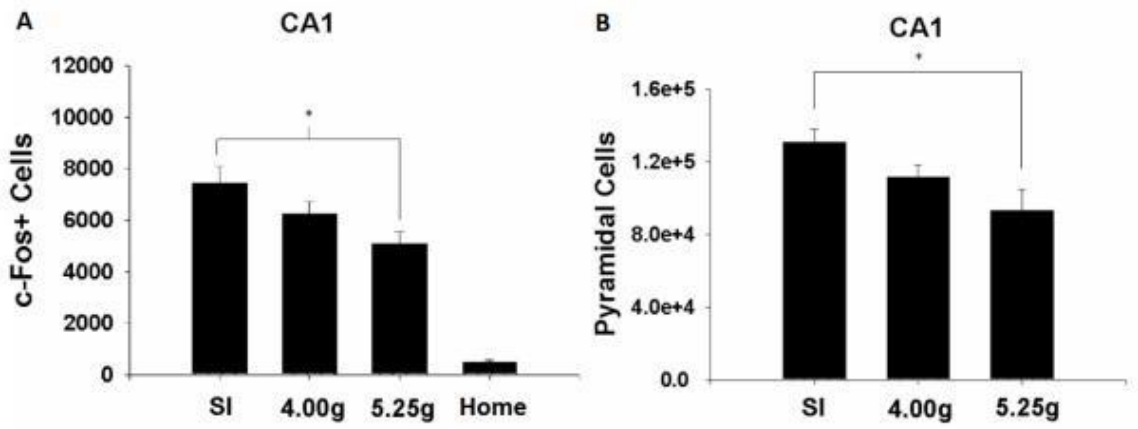


Figure 3.2 Mean estimates of CA1 c-Fos+ cells (±SE) 2h following preexposure (5 minutes of context exploration) in Groups SI, 4.00g, and 5.25g. Home rats were sacrificed from their home cage. B. Mean (±SE) CA1 pyramidal cells in rats from Groups SI, 4.00g, and 5.25g. "*" indicates significant group differences ($p<0.05$). PD4-9 alcohol exposure significantly decreased CA1 pyramidal cell counts and *c-Fos* protein expression on the preexposure day of the CPFE. Taken and adapted from Murawski et al., 2012.

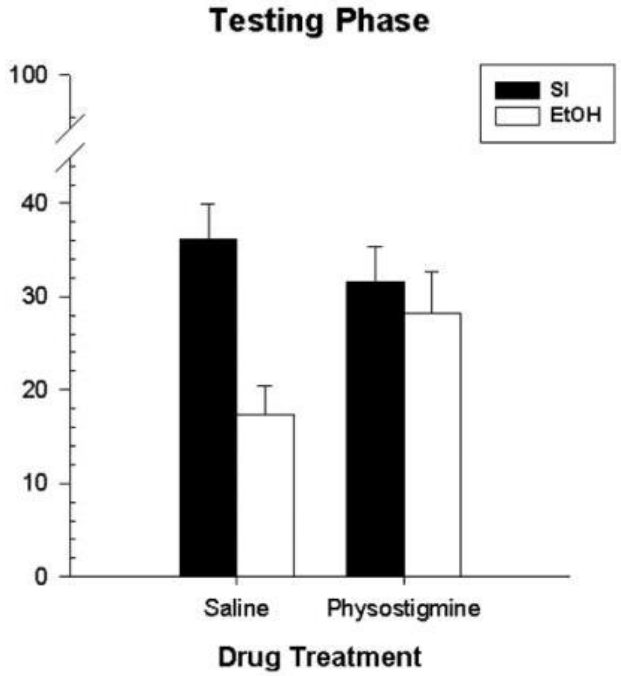


Figure 3.3 Systemic administration of the acetylcholinesterase inhibitor Physostigmine (PHY) prior to each phase of the CPFE (PD31-PD33) rescues impaired retention freezing resulting from PD7-9 EtOH exposure (5.25g/kg/day). Black bars depict preexposed sham-intubated animals given PHY or saline while white bars depict PD4-9 EtOH animals given PHY or saline. Taken and adapted from Dokovna et al., 2013.

## 3.8 Conclusions

This chapter summarized key findings highlighting prefrontal, hippocampal, cholinergic, and behavioral dysfunction in rodent models of FASDs involving third-trimester equivalent alcohol exposure. While human FASD research has recently started to emphasize prefrontal dysfunction as a feature of the disorder, animal model research has largely continued its focus on hippocampus. *One main hypothesis of the experiments in this dissertation is that functional, molecular changes in the mPFC underlie cognitive deficits arising from third-trimester equivalent alcohol exposure in rats.* Armed with recent discoveries that mPFC is critical for the CPFE (Heroux et al., 2017; Robinson-Drummer et al., 2017), this dissertation re-examines disruptions in the CPFE that our lab has traditionally attributed to disrupted hippocampal functioning in alcohol-exposed rats. In the experimental chapters of this dissertation, I explore hippocampal-prefrontal circuitry supporting contextual memory processes of the CPFE, which informs this alcohol-induced insult (see **Chapter 6**). Next, I examine the effects of neonatal alcohol exposure on activity-driven prefrontal and hippocampal gene expression during the acquisition of context memory in the CPFE (see **Chapter 7**). Finally, I examine the effects of enhancing cholinergic signaling prior to behavioral testing on CPFE performance and regional gene expression (see **Chapter 8**).

# Chapter 4

# FOUNDATIONAL STUDIES

The preceding chapters reviewed the neurobehavioral consequences of developmental alcohol exposure and the neural mechanisms of contextual fear conditioning. The present chapter describes my published research that serves as a foundation for the experiments performed in this dissertation. These studies characterize 1) the role of NMDAR plasticity in the acquisition and consolidation of contextual fear memory in the CPFE, 2) the involvement of the mPFC across variants of contextual fear conditioning, 3) the role of mPFC and dHPC cholinergic signaling in the CPFE, and 4) regional patterns of IEG induction during context and contextual fear learning in the CPFE.

## 4.1 NMDA-Receptor Cell Signaling and the CPFE

As reviewed in Chapters 1and 2, the CPFE paradigm permits separate analysis of learning about the context, acquiring a context-shock association, and retrieval/expression of that association (see **Figure 1.2**). In adult rats, context learning depends on NMDAR activity in dHPC whereas context-shock learning (during immediate-shock training) depends on NMDAR activity in BLA (Matus-Amat et al., 2007). Our lab has shown that the same is true for juvenile (PD24) and adolescent (PD31) rats (Burman, Murawski, Schiffino, Rosen, & Stanton, 2009; Miller, Heroux, & Stanton, 2019; Schiffino et al., 2011). Importantly, previous pharmacological studies using the CPFE fail to dissociate between acquisition and consolidation

processes of the CPFE. To address this, Heroux et al. (2016) examined the role of NMDAR activity across phases of the CPFE using pre- and post-phase drug administration, in addition to using post-shock freezing as a measure of contextual fear acquisition.

Heroux et al. (2016) gave adolescent rats pre- or post-phase systemic injections of the NMDAR antagonist MK-801 during context preexposure and immediate-shock training; and pre-phase MK-801 prior to retention. Administration of MK-801 prior to context preexposure or immediate-shock training abolished post-shock and retention test freezing in the CPFE. There was no difference in freezing between MK-801-injected rats preexposed to the training context [Pre group] and non-associative controls preexposed to an alternate context [Alt-Pre group]; see **Figures 4.1-4.2**). Interestingly, while any dose of MK-801 used was sufficient to disrupt retention test freezing, only the highest dose of MK-801 (0.1mg/kg) impaired post-shock freezing (see **Figure 4.2**). This demonstrates that post-shock freezing may be less sensitive to pharmacological disruption than retention test freezing. There was no effect of MK-801 when given prior to retention (data not shown), ruling out performance effects of the drug on freezing behavior. There was also no effect of MK-801 given after immediate-shock training, but a mild impairment was seen after post-preexposure injection. This indicates that effects of MK-801 on consolidation of context learning or the context-shock association are not sufficient to account for effects of the drug on these types of learning per se. Taken together, Heroux et al. (2016) demonstrated that NMDAR plasticity is required for the acquisition of the context representation and context-shock association in the CPFE. Importantly, this was the first pharmacological study to include a post-shock freezing test to dissociate the role of NMDAR activity in

the acquisition vs. consolidation of contextual fear in the CPFE. These findings provide a procedural framework both for future pharmacological studies and most of my subsequent studies, as reviewed below and presented in this dissertation.

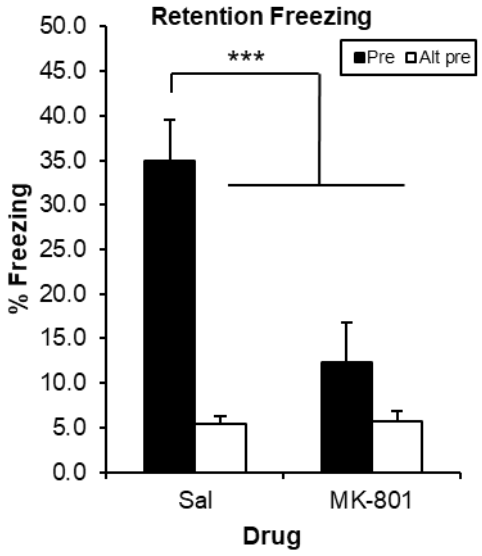


Figure 4.1 Mean percent freezing (± SEM) depicted for Pre (black bars) and Alt-Pre (white bars) groups across drug treatment conditions when the drug is administered prior to context preexposure. The CPFE was observed in a 24 hour retention test for the Saline control group but not when MK-801 was injected prior to context preexposure (***$p$<.001). Taken from Heroux et al. (2016).

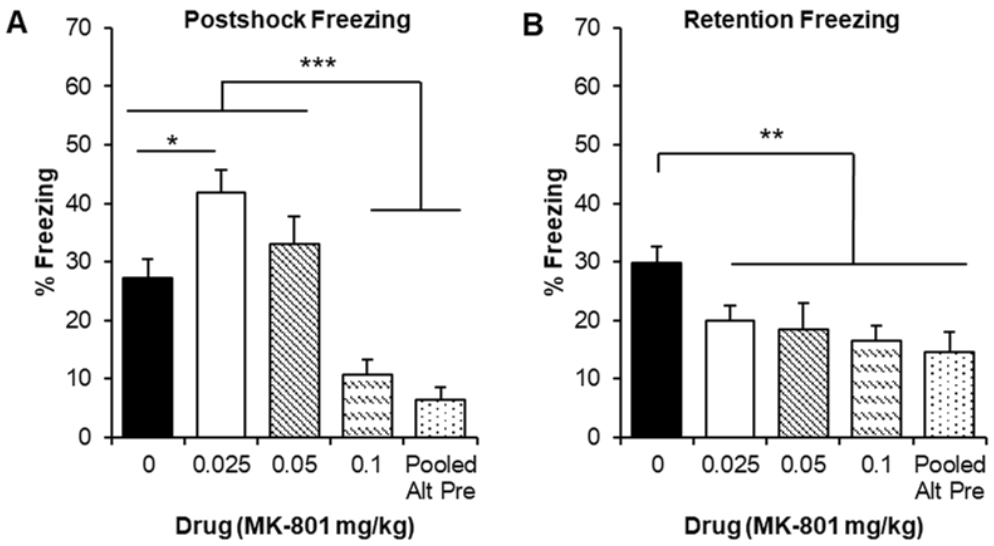


Figure 4.2 Mean percent freezing (± SEM) depicted for drug conditions when the drug is administered prior to immediate-shock training followed by a 1 minute post-shock freezing test (Panel A) and a retention freezing test 24 hours later (Panel B). Higher doses of MK-801 (0.1 mg/kg) disrupt both post-shock and retention test freezing (***$p$<.001) while lower doses of MK-801 (0.025 or 0.05 mg/kg) only disrupt retention freezing. (**$p$<.05). Alternate context preexposed rats were pooled across all drug conditions. Taken from Heroux et al. (2016).

## 4.2 Involvement of the mPFC across Variants of Contextual Fear Conditioning

As reviewed in **Chapter 2**, the role of the mPFC in fear conditioning has been traditionally attributed to the long-term, systems-level consolidation of memory and to the regulation of fear memory expression via the PL and IL sub-regions (Frankland & Bontempi, 2005; Gilmartin, Balderston, & Helmstetter, 2014; Giustino & Maren, 2015; Rozeske et al., 2015). Despite this, recent evidence suggests that the mPFC is important for processing contextual information during trace and contextual fear conditioning (see **Section 2.1.2** in **Chapter 2**). The functional role of the mPFC in supporting distinct learning and memory processes during contextual fear conditioning is unclear.

To address this, Heroux et al. (2017) examined the causal role of the mPFC in distinct learning and memory processes across phases of the CPFE and sCFC in adolescent rats. Rats were given a bilateral intra-mPFC infusion of the $GABA_A$ agonist muscimol (MUSC) or the vehicle phosphate buffered saline (PBS) prior to each phase of the CPFE or prior to training in sCFC. Inactivation via muscimol infusion during any phase of the CPFE (context preexposure, immediate-shock training, or retention) disrupted retention test freezing (see **Figures 4.3**-**4.5**). Importantly, mPFC inactivation prior to immediate-shock training did not disrupt post-shock freezing even though it abolished 24-h retention freezing in the same rats. This same infusion prior to training in sCFC had no effect on post-shock or retention test freezing (see **Figure 4.6**). These experiments demonstrate that, in the CPFE, the mPFC is required for the **1)** acquisition and/or consolidation of the conjunctive context representation, **2)** consolidation of the context-shock association, and **3)** long-term retrieval and expression of the context-shock association. The mPFC is not required for the retrieval of the context representation or its association with shock in the

CPFE. In contrast, the mPFC is not required for the acquisition or consolidation of a context-shock association in single-trial sCFC. Intact freezing in muscimol-infused rats in sCFC also rules out any possible "performance" effects of drug infusion, such as impaired feature perception, shock sensitivity, locomotion or state dependency. Taken together, these results demonstrate prefrontal-dependence of memory processes in the CPFE but not single-trial sCFC. Importantly, these experiments provide a new perspective from which to examine CPFE impairments in rats receiving neonatal alcohol exposure, which has been previously attributed solely to impaired hippocampal functioning (see **Chapter 3**).

Importantly, these studies and all of the experiments in this dissertation assess behavior in adolescent rats to help guide future research across earlier (e.g., juvenile) and later (e.g., adult) stages of ontogeny in normally-developing and alcohol-exposed rats. Our lab has shown that the CPFE develops between PD17 and PD24 in the rat, and this development has canonically been thought to depend on the maturation of the hippocampal formation (Jablonski et al., 2012; Schiffino et al., 2011). Despite this, as reviewed above, we've recently discovered a robust dependence of the CPFE on the mPFC (Heroux et al., 2017). Accordingly, these findings call into question both the neural mechanisms underlying both the ontogeny of the CPFE and the impairment of this task by neonatal alcohol exposure. Adolescence was also assessed because studies of FASD in humans emphasize developmental outcomes, e.g., in school-aged children (May et al., 2009).

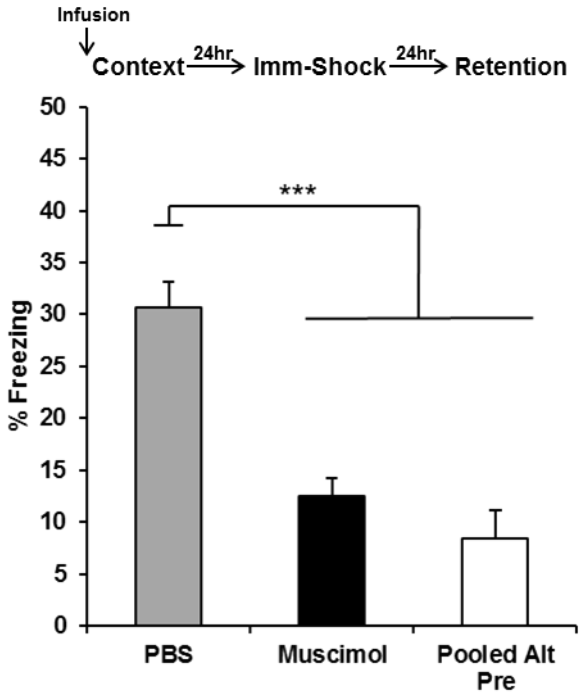


Figure 4.3 Mean percent freezing (± SEM) on the retention test day as a function of drug and behavioral treatment group. Infusion of muscimol into the mPFC prior to context preexposure reduced freezing during a retention test to the level of the non-associative controls preexposed to an alternate context (pooled across drug treatment) and significantly disrupted freezing relative to rats infused with PBS on the preexposure day (***$p$<.001). Taken from Heroux et al. (2017).

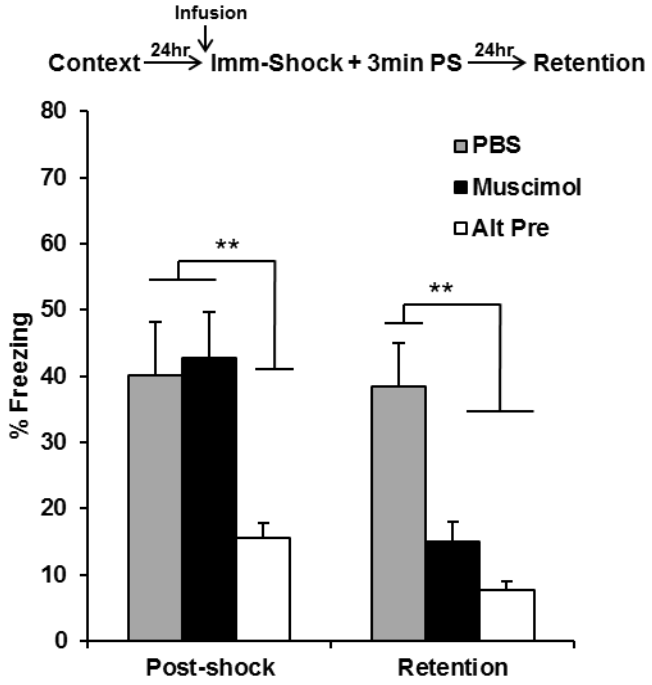


Figure 4.4 Mean percent freezing (± SEM) in an immediate 3min post-shock and 5 min 24 hr retention test depicted for rats receiving PBS or muscimol 15 min prior to context-shock training. Infusion of muscimol into the mPFC prior to context-shock training had no effect on context-fear acquisition but significantly disrupted 24hr retention test freezing relative to rats infused with PBS. (**$p < .01$). Adapted from Heroux et al. (2017).

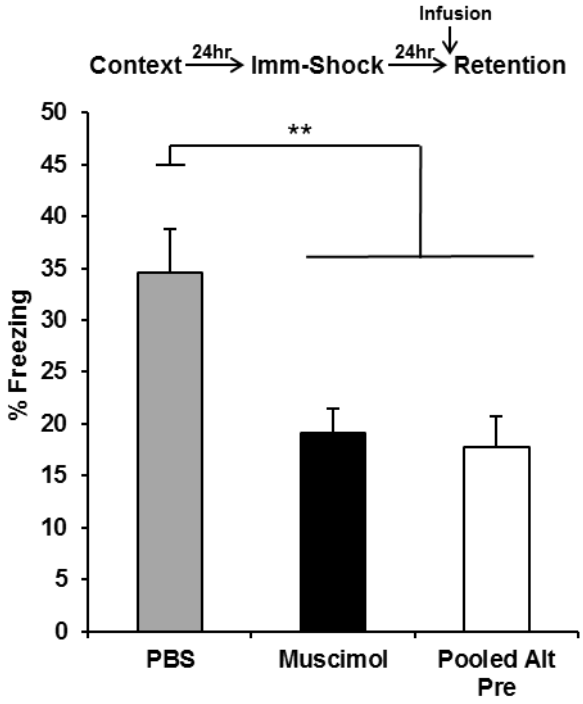


Figure 4.5 Mean percent freezing (± SEM) depicted for rats receiving PBS or muscimol 15 min prior to a 5 min retention test occurring 24hr after conditioning with no post-shock test. Infusion of muscimol into the mPFC prior to the retention test reduced freezing to a level comparable to non-associative controls (pooled across drug treatment) and significantly lower than rats infused with PBS (**$p$<.01). Taken from Heroux et al. (2017).

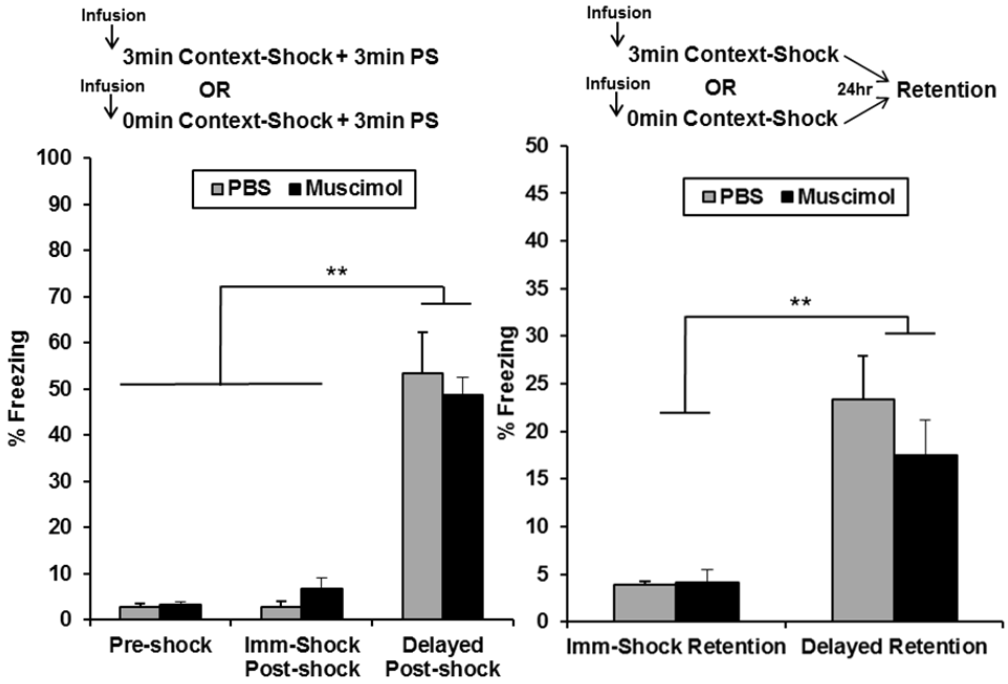


Figure 4.6 Mean percent freezing (± SEM) in a between-subjects 3min post-shock test (left panel) or 24hr retention test (right panel) depicted for rats receiving PBS or muscimol 15 min prior to conditioning in sCFC (note change in y-axis scale across panels). Rats received either 3min of context exposure (*Delayed-Shock* condition) or no context exposure (*Imm-Shock* condition) prior to the two foot-shocks. The Pre-shock bar represents freezing during the 3 minutes of context exposure before the foot-shocks. Infusion of muscimol into the mPFC prior to conditioning had no effect on freezing behavior measured in the post-shock or retention tests (*p*s > .80). Rats receiving context exposure during conditioning (*Delayed-Shock*) froze significantly more than rats receiving no context exposure prior to the shock (Imm-Shock controls, *Imm-Shock*; ***p* < .01). Taken from Heroux et al. (2017).

## 4.3 Role of mPFC and dHPC Muscarinic-Receptor Activity in the CPFE

As reviewed in **Chapter 3**, developmental alcohol exposure disrupts development of brain and cognition, and disrupted cholinergic activity is especially implicated in these effects. Our lab has shown that enhancing cholinergic signaling by

systemic administration of PHY rescues impaired retention test freezing in the CPFE (Dokovna et al., 2013). The effects of PHY might be due to enhanced muscarinic-receptor signaling, as prior work has demonstrated that systemic muscarinic-receptor antagonism disrupts both sCFC and the CPFE (Anagnostaras, Maren, & Fanselow, 1995; Anagnostaras, Maren, Sage, Goodrich, & Fanselow, 1999; Brown, Kennard, Sherer, Comalli, & Woodruff-Pak, 2011; Hunt & Richardson, 2007). These findings led us to examine the functional role of prefrontal and hippocampal cholinergic muscarinic-receptor activity across phases of the CPFE (Robinson-Drummer et al., 2016, 2017). We administered the muscarinic-receptor antagonist scopolamine (SCOP) systemically or into dHPC prior to all phases or each individual phase of the CPFE in adolescent rats. All of these manipulations disrupted retention test freezing. For example, the Pre-SCOP group froze at the same level as the Alt-Pre control group (pooled across drug) and at lower levels than vehicle (Pre-PBS) or no-surgery controls (Pre-UND; Robinson-Drummer et al., 2016; see **Figure 4.7**). Similar SCOP infusions delivered into the mPFC disrupted retention test freezing when given prior to context preexposure, immediate-shock training, or all three phases, but not the retention phase (Robinson-Drummer et al., 2017). Taken together, these results demonstrate that muscarinic-receptor signaling in both the mPFC and dHPC is crucial for both context learning and contextual fear acquisition and/or consolidation in the CPFE. These results inform our lab's previous findings that physostigmine rescues retention test freezing in rats receiving neonatal alcohol exposure from PD7-9 (Dokovna et al., 2013), suggesting that developmental alcohol exposure may impair the CPFE by disrupting prefrontal and/or hippocampal cholinergic signaling during behavior.

Collectively, this work serves as an empirical foundation for **Chapter 8** of this dissertation.

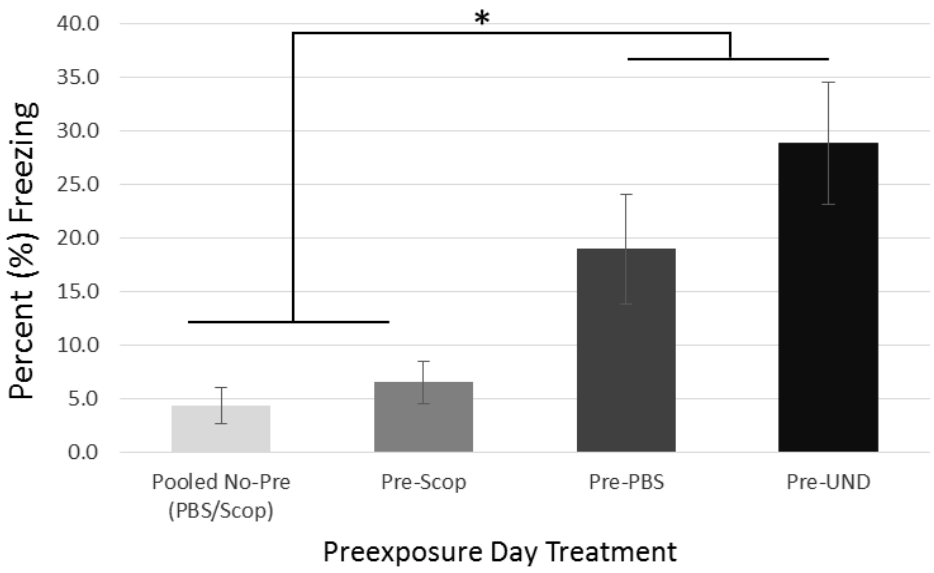


Figure 4.7 Mean (± SEM) percent test freezing as a function of group (No-Pre, Pre-Scop, Pre-PBS and Pre-UND). The undisturbed group (UND) were rats that received surgery but no Scop infusion. Pre rats (Pre-Scop, Pre-PBS and Pre-UND) were preexposed to the training and testing context on preexposure day. No-Pre rats (pooled from Scop and PBS subgroups) were preexposed to the alternate context, however training and testing were in the same context as the Pre groups. The Pre-UND and Pre-PBS groups were significantly different from the (pooled) No-Pre and Pre-Scop ($p < 0.05$). Overall, intra-dHPC scopolamine impaired the CPFE when administered prior to the preexposure day. This same effect was seen when infusions were done prior to training or testing. Taken from Robinson-Drummer et al. (2016).

## 4.4 Regional Expression of Immediate Early Genes across Phases of the CPFE

Our lab has shown that context preexposure and immediate-shock training differentially induce the expression of the IEG *Egr-1* in sub-regions of the mPFC, dHPC, and LA (Asok et al., 2013; Chakraborty et al., 2016; Robinson-Drummer et al., 2018; Schreiber et al., 2014; Chapter 2). Most interesting is the finding that immediate-shock training induces learning-related expression of *Egr-1* in the mPFC in

adolescent and adult rats. As reviewed in Chapter 1, the CPFE emerges between PD17 and PD24 in rats (Jablonski et al., 2012; Schiffino et al., 2011). Accordingly, we hypothesized that experience- and learning-related *Egr-1* expression in the prefrontal cortex may correlate or even cause the ontogeny of the CPFE. To test this, we analyzed freezing performance and *Egr-1* expression during context preexposure and immediate-shock training in PD17 and PD24 rats (Robinson-Drummer et al., 2018). PD17 rats failed to show a significant CPFE compared to PD24 rats regardless of single or multiple exposures to the training context. Moreover, PD24 rats showed an elevation in prefrontal, hippocampal, and amygdalar *Egr-1* expression during context preexposure that was absent in PD17 rats. Interestingly, using a single context exposure, training-day *Egr-1* expression was not different between PD17 and PD24 rats and was not related to learning. These results showed that PD24 rats show the CPFE even in the absence of any observed learning-related *Egr-1* expression in the prefrontal cortex during training (Robinson-Drummer et al., 2018). Ultimately, this suggests that preexposure but not training-day *Egr-1* expression may underlie the ontogeny of the CPFE.

To extend this research to other IEGs, Heroux et al. (2018) examined expression of *c-Fos*, *Arc*, *Egr-1*, and *Npas4* during different phases of the CPFE in adolescent rats, for reasons described previously (see Chapter 2, Section 2.2.3). In this study, IEG expression was analyzed in the mPFC, dHPC, and BLA during context preexposure and immediate-shock training (with or without a post-shock freezing test) via quantitative real-time polymerase chain reaction (qPCR) on RNA extracted from dissected tissue. Context preexposure to the training (Pre group) or alternate context (Alt-Pre group) identically induced the expression of every IEG in all three regions

above behaviorally-naïve homecage control rats (HC group; see **Figure 4.8B-D**). Immediate-shock followed by a post-shock freezing test increased mPFC *c-Fos* expression in the Pre group above the Alt-Pre group, indicating expression related to associative learning. This was not seen with other IEGs in mPFC or with any IEG in dHPC or BLA (data not shown). Finally, when the post-shock freezing test was omitted, training-related increases were observed in prefrontal *c-Fos*, *Arc*, *Egr-1*, and *Npas4*, hippocampal *c-Fos*, and amygdalar *Egr-1* expression (see **Figure 4.9B-D**). Two main conclusions can be drawn from these results: **1)** prefrontal activity and/or plasticity likely plays an important role in memory processes in the CPFE (i.e., extending beyond *Egr-1* expression); and **2)** context exposure during a post-shock freezing test induces IEG expression that may obscure learning-related expression during contextual fear conditioning.

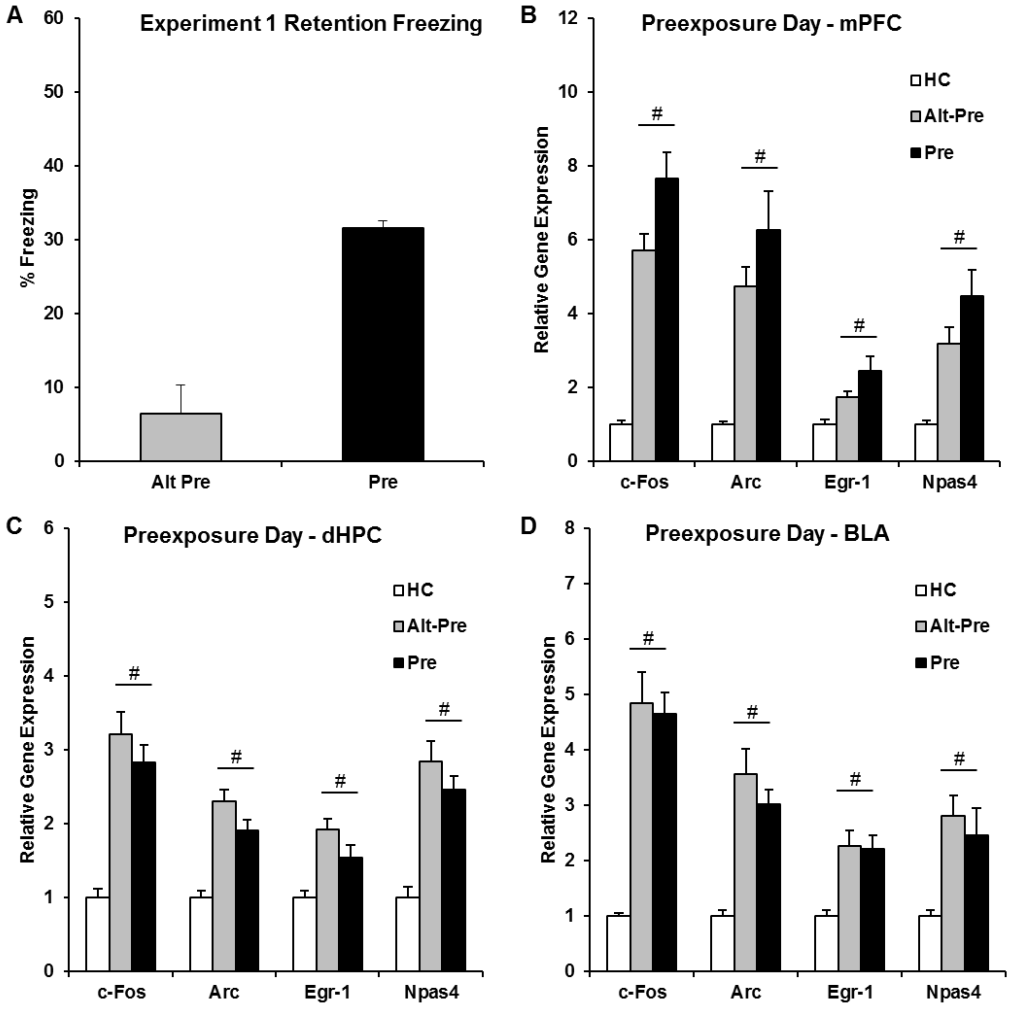


Figure 4.8 Behavioral data confirming CPFE is present in unsacrificed littermates (**A**) and post-context-preexposure IEG expression in the mPFC (**B**), dHPC (**C**), and BLA (**D**) for the HC, Alt-Pre, and Pre experimental groups. (**A**) The Pre group froze significantly more than the Alt-Pre group during the 5 min retention test (Alt-Pre, $p < .001$). (**B**-**D**). Context exposure on the preexposure day of the CPFE significantly induced the expression of *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in every region, with Pre and Alt-Pre gene expression elevated above HC ($p$s > .01). # indicates a significant elevation above HC. No differences were found between Alt-Pre and Pre groups.

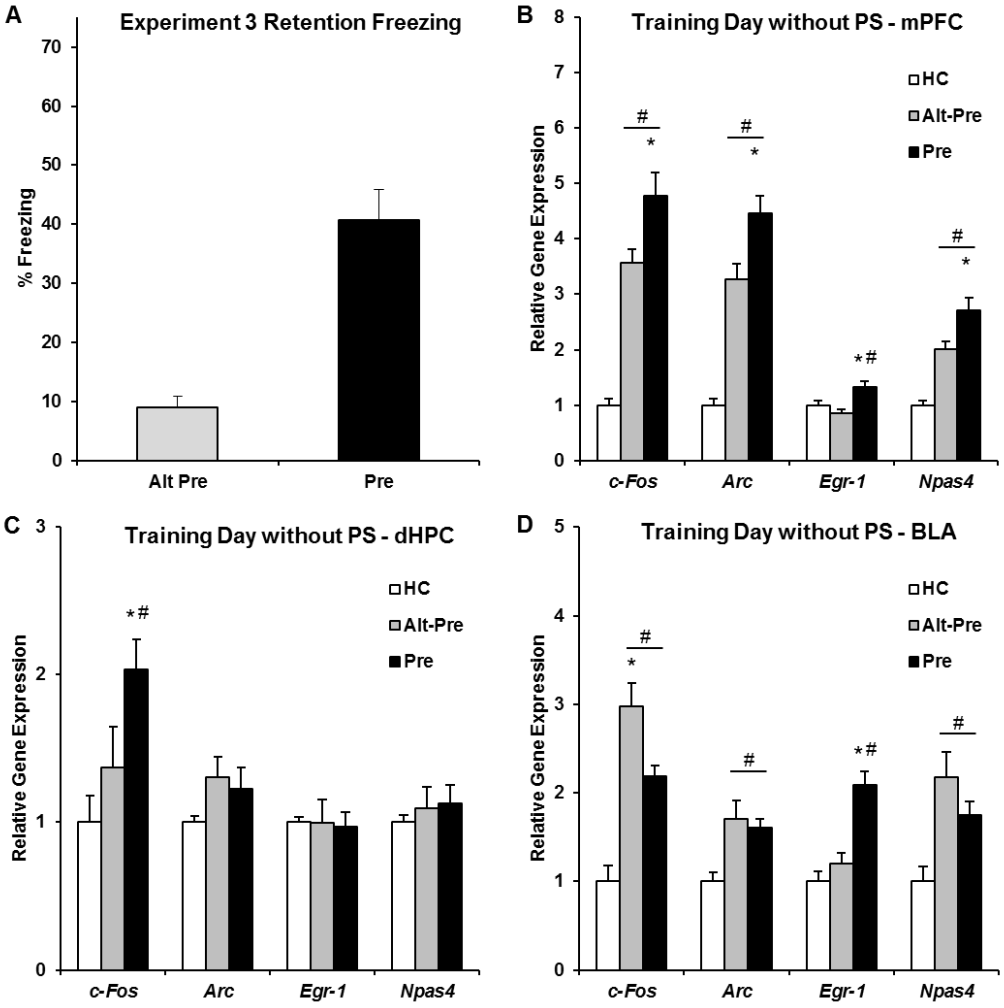


Figure 4.9 Behavioral data confirming CPFE in unsacrificed littermates (**A**) and post-training (without a post-shock freezing test) IEG expression in the mPFC (**B**), dHPC (**C**), and BLA (**D**) for the HC, Alt-Pre, and Pre experimental groups. (**A**) The Pre group froze significantly higher than the Alt-Pre group during the 5 min retention test (Alt-Pre, $p < .001$). (**B**) Immediate-shock training significantly induced mPFC *c-Fos*, *Arc*, *Npas4* expression in the Pre and Alt-Pre groups above HC controls ($ps < .001$), with an associative increase in all four genes (i.e., including *Egr-1*; $p<.01$). (**C**) dHPC *c-Fos* expression in the Pre group was significantly elevated above both Alt-Pre and HC ($p < .04$). (**D**) BLA *c-Fos*, *Arc*, and *Npas4* expression in the Pre and Alt-Pre groups was significantly elevated above HC ($ps < .05$), with an additional associative increase in *Egr-1* expression ($p < .05$). **#** indicates significant elevation above HC; ***** indicates a significant difference between Pre and Alt-Pre.

## 4.5 Conclusions

The experiments reviewed above establish a foundation and framework with which to examine prefrontal and hippocampal involvement in context memory (**Chapter 6**), and the neurobehavioral mechanisms of impaired cognition in our rat model of FASD (**Chapters 7 and 8**). These studies established: **1)** the role of NMDAR plasticity and use of post-shock freezing as a measure of fear acquisition in pharmacological and molecular studies of the CPFE, **2)** the prefrontal dependence of memory processes in the CPFE but not sCFC, **3)** the involvement of prefrontal and hippocampal cholinergic signaling in supporting the CPFE, and **4)** the regional patterns of expression of multiple different IEGs across phases of the CPFE. Taken together, these studies demonstrated prefrontal, glutamatergic, and cholinergic dependence of the CPFE, which will provide novel insight into CPFE impairments seen after neonatal alcohol exposure in our rat model of FASD.

# Chapter 5

# GENERAL METHODS

The present chapter describes the general methods used throughout the experiments in this dissertation. Experimental procedures that are unique to or altered in particular experiments are noted in the respective method section for that experiment. The descriptions below are based on previously published descriptions (Dokovna et al., 2013; Heroux, Horgan, Rosen, et al., 2019; Heroux et al., 2018, 2016, 2017; Heroux, Robinson-Drummer, et al., 2019; Murawski & Stanton, 2011).

## 5.1 Material and Methods

### 5.1.1 Animal Subjects

Rats were derived from litters bred by the Office of Laboratory Animal Medicine at the University of Delaware. Time-mated females were housed with breeder males overnight and, if an ejaculatory plug was found the following morning that day was designated as gestational day (GD) 0. Dams were housed in clear polypropylene cages (45 cm × 24 cm × 21 cm) with standard bedding and access to ad libitum water and rat chow. Rats 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. Litters were culled on PD3 to eight pups (4 males and 4 females when possible) and were paw-marked with subcutaneous injections of non-toxic black ink for later identification. Pups were weaned from their mother on PD21 and housed with same-sex littermates in clear cages (45 cm × 24 cm × 17 cm). On PD29, rats were individually housed in

clear cages (30 cm × 18 cm × 17 cm) with ad libitum access to water and rat chow for the remainder of the experiment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Delaware following guidelines established by the National Institute of Health.

### 5.1.2 Stereotaxic Surgery, Drug Infusion, and Histology

This section describes stereotaxic surgery, intra-cranial drug infusion, histological procedures used in both experiments in **Chapter 6**.

#### 5.1.2.1 Stereotaxic Surgery

Surgical implantation of intracranial cannula in the mPFC and vHPC for CPFE behavioral experiments has been previously done in adolescent rats (Heroux et al., 2017; Robinson-Drummer, Heroux, & Stanton, 2017) and adult rats (Rudy & Matus-Amat, 2005), respectively. On PD29, rats were anesthetized with an i.p. injection of 1 mg/kg of an 85:15 ketamine/xylazine drug mixture prior to surgery. In Experiment 6.1, guide cannulas (Plastics One, Roanoke, VA) were bilaterally implanted to terminate in the mPFC using the following coordinates: anteroposterior (AP) +9.0 mm, mediolateral (ML), ±0.6 mm relative to interaural midline, and dorsoventral (DV), -2.3 mm relative to the top of the skull. In Experiment 6.2, cannulas were bilaterally implanted in the vHPC using the following coordinates: AP +1.9 mm, ML ±4.7 mm relative to interaural midline, and DV -5.3 mm relative to the top of the skull. Twenty-four hours following surgery (PD30), rats were infused with 0.25-0.50 µl of the vehicle PBS in both hemispheres to reduce occlusion in the guide cannula and to acclimate the rats to being handled during infusions before the start of behavioral procedures the following day (PD31).

#### 5.1.2.2 Intra-mPFC and Intra-vHPC Drug Infusions

In Experiments 6.1 and 6.2, microinjections of the vehicle PBS or the $GABA_A$ receptor agonist muscimol (Sigma-Aldrich, St. Louis, MO) were administered approximately 15 minutes prior to context preexposure on PD31. In Experiment 6.1, mPFC-cannulated rats were infused with .25 μl/side of PBS or muscimol (0.5 μg) over 1 min, and injector tips were left in the guide cannula for one minute following infusion to allow sufficient diffusion of the drug. Previous studies from our lab have used this dose to inactivate the mPFC during the CPFE, with additional analyses confirming drug spread using fluorescent muscimol (Heroux et al., 2017; **Section 4.2** above). In Experiment 6.2, vHPC-cannulated rats were infused with .5 μl/side of PBS or muscimol (0.5 μg) over 1 min, with injectors left in for 1 min after infusion (Rudy & Matus-Amat, 2005). Rats were returned to their home-cage for approximately 15 minutes until the start of behavioral testing.

#### 5.1.2.3 Histology

Rats in the IEG group were rapidly decapitated without anesthesia 30 min after context preexposure. In Experiments 6.1 and 6.2, half of the brain was used to verify cannula placements, and coronal brain slabs were cut out the other half for IEG analyses. The methods for tissue dissection and IEG analysis can be seen in Section 5.1.5. In the rats that underwent the full CPFE procedure, rats were sacrificed by rapid decapitation after testing, with brains removed and frozen in -45°C isopentane and then stored at -80°C before being sectioned on a microtome. Sagittal and coronal sections (50-60 μm) were taken from the gene expression and behavior sampling groups, respectively. Slides were photo-captured and analyzed to confirm the placement of the cannula injector tip in the mPFC.

### 5.1.3 Animal Model of Fetal Alcohol Spectrum Disorder

This section describes neonatal alcohol dosing procedures and blood alcohol concentration analyses that were used across experiments in Chapters 7 and 8.

#### 5.1.3.1 Neonatal Alcohol Dosing (PD4-9, 5.25g/kg/d)

Neonatal ethanol dosing via intragastric intubation occurred over PD4-9. Littermates were randomly assigned to receive either ethanol (EtOH group) or sham intubations (SI group), with an equal number of males and females in each litter whenever possible. Same-sex littermates assigned to the same dosing condition (EtOH or SI) were assigned to different experimental groups so that no more than one same-sex littermate was assigned to any particular condition. Briefly, on PD4, pups were separated from their mothers and placed into weigh boats set over a heating pad that provided warmth during the separation. Pups were weighed prior to the first intubation session (occurring daily at 9am ± 1hr). The intubation process involved passing PE10 tubing lubricated with corn oil down the esophagus and into the stomach of the rat pup. Rats in the SI group received intragastric intubations on the same schedule as the EtOH group, and the tube was removed after approximately 6-8 seconds during each scheduled intubation without the infusion of any solution. Rats in the EtOH group were intubated and given a daily dose of 5.25 g/kg of alcohol, [11.9% v/v ethanol (made from 95% ethanol)] in a custom milk formula previously described (Kelly & Lawrence, 2008). This dose was divided into two feedings each day, separated by 2hr. The formula was delivered in a volume of 0.02778 ml⁄g body weight. A third intubation of the milk formula (containing no ethanol) was administered two hours after the second daily alcohol dosing. After each intubation was completed (<20 minutes per litter), pups were returned as a litter to their mothers. Importantly,

although intragastric intubation of alcohol using these parameters results in a transient reduction in body weight and growth (Jablonski & Stanton, 2014; Kelly & Lawrence, 2008; Murawski & Stanton, 2011), this reduction is absent by adolescence and thus likely does not contribute to behavioral deficits resulting from exposure. Importantly, this neonatal exposure produces high BACs (~400 mg/dl) modeling severe binge drinking in the rat model; while manipulation is not possible, BACs of over 200 mg/dl are commonly associated with later development of FAS in the human condition (see Patten et al., 2014 for full discussion).

#### 5.1.3.2 Blood Alcohol Concentrations (BACs)

On PD4, 90 min following the second alcohol intubation, pups received a small tail-clip and a 20µl blood sample was collected using a capillary tube. Blood samples from Group SI were discarded and those from alcohol-exposed pups were saved for further blood alcohol analysis. Blood samples from alcohol-exposed pups were centrifuged, and the plasma was collected and stored at -20°C. Blood alcohol concentrations were determined using an Analox GL5 Analyzer (Analox Instruments, Luneburg, MA) by measuring the rate of oxidation of alcohol in each plasma sample. BACs (expressed in mg⁄dl) were calculated based on comparisons to known values of an alcohol standard solution.

### 5.1.4 Behavioral Equipment and Procedures

This section describes the apparatus, stimuli, and behavioral procedures for the CPFE used across experiments in **Chapters 6, 7, and 8** and sCFC procedures used in **Chapter 7**.

#### 5.1.4.1 Apparatus, Stimuli, and Freezing Software Analyses

The apparatus and stimuli used have been previously described (Heroux et al., 2016, 2017; Murawski & Stanton, 2010; Robinson-Drummer et al., 2016). Fear conditioning occurred in four clear Plexiglas chambers measuring 16.5 cm × 12.1 cm × 21.6 cm, which were arranged in a 2 × 2 formation on a Plexiglas stand within a fume hood to provide ambient light and background noise (Context A). Each chamber had a grid floor made of nine stainless steel bars (11.5 cm from the top of the chamber), 0.5 cm in diameter and spaced 1.25 cm apart. The alternate context (Context B) consisted of the same Plexiglas chambers with a convex wire mesh insert that covered the back wall and floor of the chamber and a white paper sleeve that covered the outside walls of the chamber. Except where noted (see Chapter 7), the unconditioned stimuli (US) were two, 1.5 mA foot-shocks, each 2s in duration, and presented 1s apart. These were delivered using a shock scrambler (Med Associates, Georgia, VT-ENV-414S) connected to the grid floor of the chamber. The fear chambers were cleaned with 5% ammonium hydroxide solution prior to each load of experimental rats.

Videos of each session (preexposure, training, testing) were recorded using Freeze Frame 3.0 software (Actimetrics, Wilmette IL) with freezing defined as a bout of 0.75 s or longer without a change in video pixilation. A human observer blind to the experimental groups verified the freezing threshold setting with Freeze View 3.0 (Actimetrics, Wilmette IL). The software program computes a "motion index" that was adjusted to set a freezing threshold separately for each animal (per software instructions) by a blind observer who verified from the video record whether small movements were scored as freezing. Once set, the threshold did not change during a session. We have validated this procedure against other scoring methods (e.g., hand

scoring of video records by two blind observers). Freezing behavior was scored as the total percent time spent freezing longer than .75s bins (defined as the cessation of all movement except breathing) in each respective session bin (context exposure, post-shock freezing, and a 24 h retention test). The data were imported into STATISTICA 64 data analysis software and freezing behavior was analyzed.

#### 5.1.4.2 *Context Preexposure Facilitation Effect* (CPFE)

The CPFE procedure consisted of three phases (context preexposure, immediate-shock training, retention testing) and took place over the course of three days from PD31 to PD33. Rats were assigned to either preexposure condition (Pre group), alternate preexposure condition (Alt-Pre group), or when applicable, a behaviorally naïve home-cage condition (HC group) that serves to establish baseline gene expression for experiments that include IEG analyses. Rats in the Pre group received exposure to Context A, the training context, while rats in the Alt-Pre group received exposure to Context B (see section 5.1.4.1). Alt-Pre rats serve as non-associative behavioral controls as they demonstrate the immediate-shock deficit (ISD), which reflects an inability to form a context-shock association without prior exposure to Context A (Fanselow, 1990).

On PD31, rats were placed in Context A or B and underwent multiple context preexposure, consisting of one initial 5 min exposure to the chamber, followed by five 1 min exposures, with a 1 min interval between exposures. In experiments that include IEG analyses, a subset of rats in the Pre and HC groups were sacrificed via live decapitation and tissue was collected for RNA extraction and qPCR 30 min after context preexposure. Alt-Pre rats are not included in IEG designs as there is no difference between Pre and Alt-Pre gene expression on the preexposure day of the

CPFE (Heroux et al., 2018, **Figure 4.9**, above). On PD32, rats in the behavior group (i.e., not sacrificed after context preexposure) were carried into the testing room, placed in their respective Context A training chamber, and within 3s, received two 1.5mA foot-shocks separated by 1s. In most cases, foot-shocks were followed by a 3-min post-shock freezing test to assess fear acquisition. Otherwise, this test was omitted. In either case, at the end of training, rats were returned immediately to their transport cages and within 1-2 minutes returned to their home cages in the colony room. On PD33, rats were returned to the same Context A chamber in which they were trained for a 5 min retention freezing test.

#### 5.1.4.3 Standard Contextual Fear Conditioning (sCFC)

The sCFC procedure was used in Experiment 7.3 in **Chapter 7**. The sCFC procedure took place over the course of two days from PD31 to PD32. All chambers, stimuli, and drug infusion protocols used were identical to the ones used in Context A for the CPFE experiments (see **Section 5.1.4.2**). On PD31, animals were assigned to either the Delayed-shock or Imm-Shock control condition. Animals in the Delayed-shock condition received three minutes of context exposure in Context A, followed by two 1.5 mA 2s foot-shocks separated by 1s. Animals in the Imm-Shock conditions were given two foot-shocks without any context exposure. This group served as behavioral controls for the delayed-shock conditions as the placement-to-shock interval was under 5 sec resulting in the immediate-shock deficit (Fanselow, 1990). Rats were removed immediately after conditioning without any post-shock freezing test. On PD32, rats in both retention conditions were tested in Context A for 5 min in the same chamber in which training occurred.

### 5.1.5 IEG Assays

This section describes the procedures and analyses by which mRNA expression of the IEGs *c-Fos*, *Arc*, *Egr-1*, and *Npas4* was determined in each IEG experiment.

#### 5.1.5.1 Brain Removal and Tissue Dissection

Rats were taken from their home cages 30 min after context preexposure and rapidly decapitated without anesthesia. Brains were removed and bathed in ice-cold saline for about 5-8 sec to increase tissue firmness. Coronal brain slabs (1-1.5 mm) were cut out using a .5mm coronal rat brain matrix. The mPFC, dHPC, vHPC, and VMT were dissected out of the coronal slabs, checking both sides for anterior-posterior boundaries. The VMT was only taken in Experiments 6.1 and 6.2. Dissection boundaries were approximately as follows (from bregma): mPFC, +4.20mm to +2.52 mm; dHPC, 2.16 mm to -3.84 mm; vHPC, -4.56 mm to -6.12 mm; and VMT, -1.92 mm to -3.24 mm (using the Paxinos & Watson [2017] rat brain atlas as a guide). Representative images for the dissections for the mPFC and dHPC can be seen in Figure 5.1 and VMT and vHPC in Figure 5.2. Dissected tissue was flash frozen on dry ice and subsequently stored at -80°C until the time of analysis. In Experiments 6.1 and 6.2, half of the brain was used to verify cannula placements, and coronal brain slabs were cut out the other half for IEG analyses (left and right sides counterbalanced across rats).

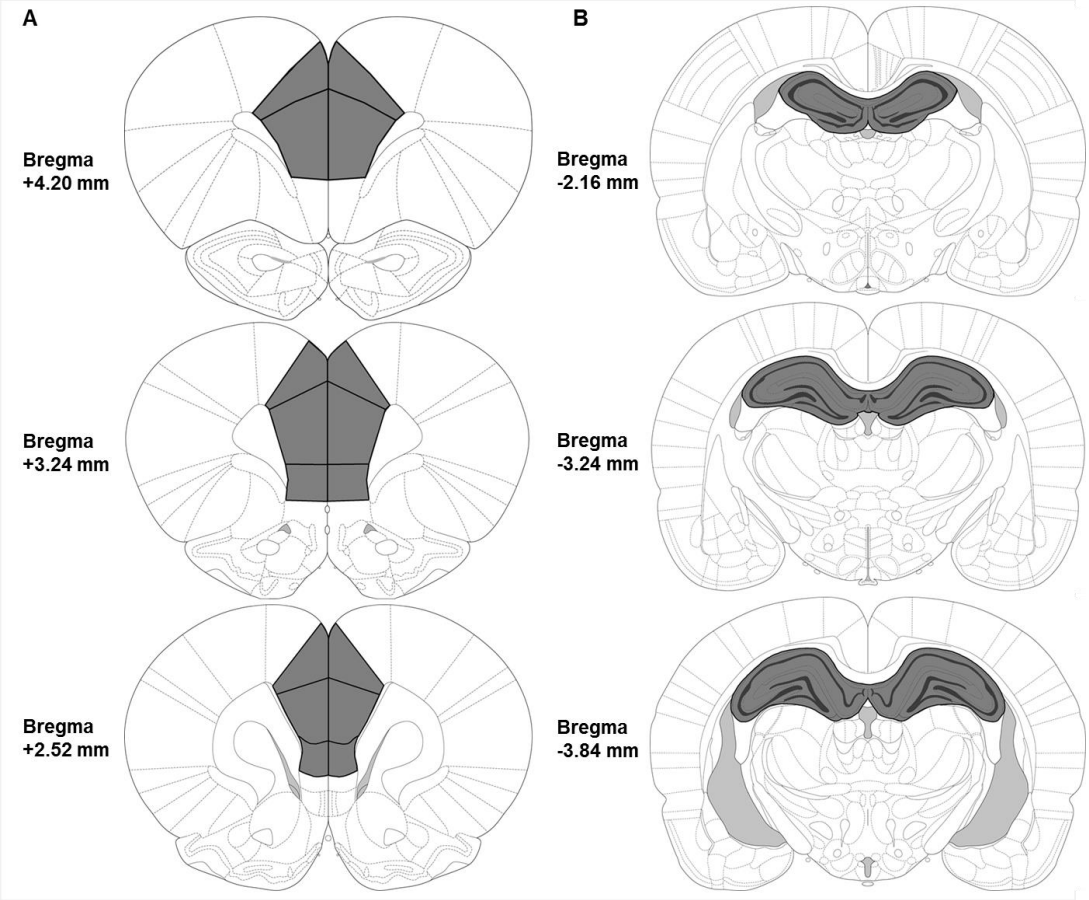


Figure 5.1 Illustration of brain regions analyzed (A, Left: mPFC; B, Right: dHPC), with dissected regions outlined in black and shaded in dark gray. Tissue from the mPFC was collected between approximately +4.20 mm to +2.52 mm relative to bregma; tissue from the dHPC was collected between about -2.16 mm to -3.84 mm relative to bregma. Images are adapted from The Rat Brain in Stereotaxic Coordinates, 6th Ed (Paxinos & Watson, 2007).

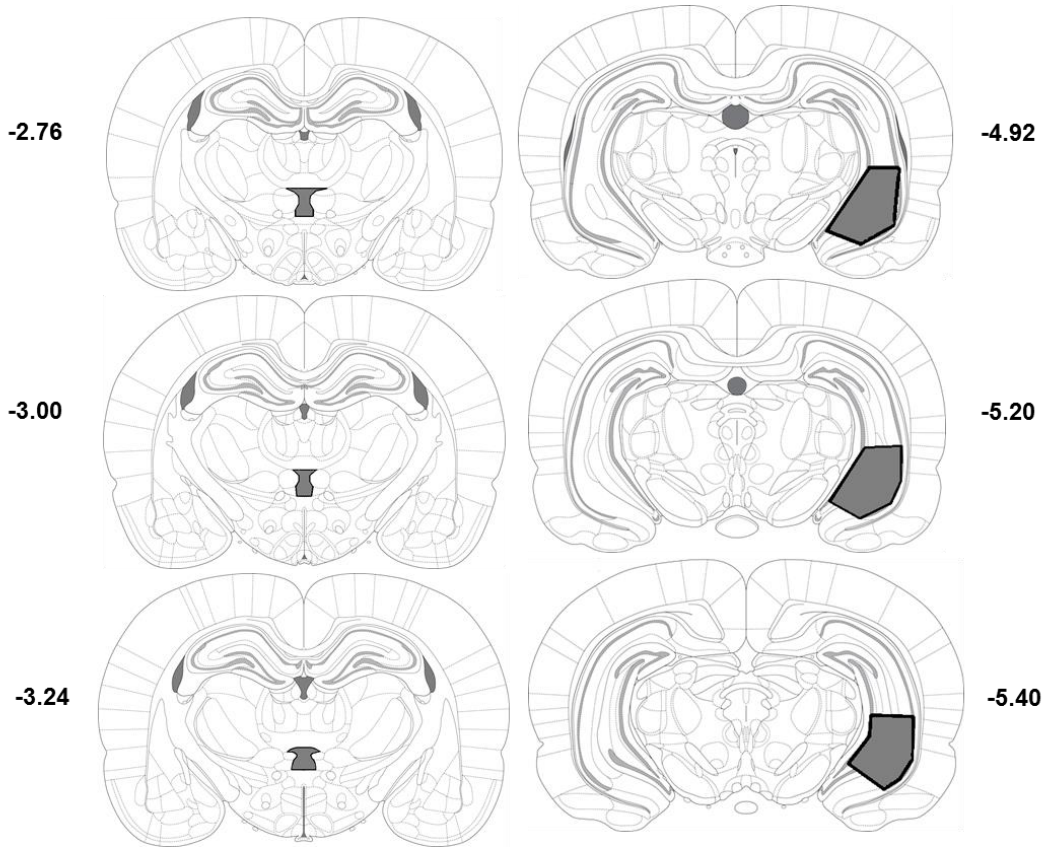


Figure 5.2 Illustration of brain regions analyzed (A, Left: VMT; B, Right: vHPC), with dissected regions outlined in black and shaded in dark gray. Tissue from the VMT was collected between approximately -1.92 mm to -3.24 mm relative to bregma; tissue from the vHPC was collected between about -4.56 mm to -6.12 mm relative to bregma. Images are adapted from The Rat Brain in Stereotaxic Coordinates, 6th Ed (Paxinos & Watson, 2007).

#### 5.1.5.2 Quantitative Real-time PCR (qPCR)

RNA was extracted from frozen tissue samples using TRIzol Reagent (Cat. No. 15596018, Invitrogen). Genomic DNA was eliminated and cDNA was synthesized from extracted RNA (1000ng/µL) using the QuantiTect® Reverse Transcription Kit (Cat. No. 205314, Qiagen). Relative gene expression was quantified by real-time PCR using the GREEN FASTMIX PERFECTA-SYBR Kit (Cat. No. 101414-270, Quantabio) in 10µL reactions on a CFX96Touch real time PCR machine. Expression of *Egr-1* was analyzed using a QuantiTect® Primer Assay (Cat. No. QT00182896, Qiagen) and diluted according to protocol. All other primers were ordered through Integrated DNA Technologies and diluted to a final concentration of 0.13 µM (*18s*, *Arc*, *c-Fos*, and *Npas-4*). The gene *18s* was used as a control for all experimental groups because it is a ribosomal housekeeping gene unchanged across any groups or manipulations. Samples were numbered, blinded to treatment group and run in duplicate on real-time PCR plates. For each reaction, the average quantitative threshold amplification cycle number ($C_q$) value was determined from each duplicate, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression for each gene relative to *18s*. The relative gene expression value was obtained by normalizing the data to the reference gene (*18s*) and to the average delta $C_T$ of the home-cage control group for each gene.

### 5.1.6 Statistical Analyses

STATISTICA 13 (Statsoft, Tulsa, Oklahoma) was used for all statistical analyses across all experiments. Analysis of behavioral and IEG data has been previously described (Heroux et al., 2018; Heroux, Robinson-Drummer, et al., 2019). In both IEG and behavioral data, rats were excluded from analysis as an outlier if they

had a score of ± 1.96 standard deviations from the group mean, but average z-scores of removed outliers historically range much higher as reported for each experiment (below). Specific experimental designs, analyses, and outlier removal can be found in the method section of each experiment. When applicable, post-hoc contrasts were performed with Newman-Keuls or Dunnett's test to assess any significant main effect or interactions revealed by the ANOVAs in each design.

# Chapter 6

# MEDIAL PREFRONTAL AND VENTRAL HIPPOCAMPAL CONTRIBUTIONS TO INCIDENTAL CONTEXT LEARNING AND MEMORY IN ADOLESCENT RATS

## 6.1 Introduction

This chapter was recently published in *Neurobiology of Learning and Memory* (Heroux, Horgan, Pinizzotto, et al., 2019), and the text appears here in adapted form. As noted in **Chapters 2 and 4**, our lab has recently discovered a novel role of the mPFC in the acquisition and/or consolidation of a conjunctive context representation in the CPFE. We hypothesized that the PFC interacts with the HPC during context learning to support these processes (see **Section 2.1.3** in **Chapter 2**). Therefore, the purpose of the current study was to further characterize regional neurobiological mechanisms underlying incidental context learning during the preexposure phase of the CPFE. We examined the effects of inactivating the mPFC (Experiment 6.1) or vHPC (Experiment 6.2) during context preexposure on expression of the IEGs *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC, dHPC, vHPC, and VMT. Across both experiments, we hypothesized that inactivation of either structure would interfere with the acquisition and/or consolidation the context representation, resulting in abolished post-shock and retention test freezing in the CPFE. Given that neither of these structures have an exclusive role in context memory, we hypothesized that inactivation of either structure would interfere with activity and thus gene expression in the other region and additional connected regions engaged by context exposure (e.g., the dHPC

and VMT). By using the CPFE to isolate distinct processes of CFC, these experiments provide novel evidence for the involvement of the mPFC and vHPC in incidental context learning and memory.

## 6.2 Materials and Methods

### 6.2.1 Subjects

Animal husbandry was as described as in **Chapter 5**. Across both experiments, there was a total of 155 adolescent (PD31) Long Evans rats (78 females and 77 males), derived from 39 separate litters bred by the Office of Laboratory Animal Medicine at the University of Delaware.

### 6.2.2 Stereotaxic Surgery

The methods for stereotaxic surgery and drug infusion are as described in **Chapter 5**. Briefly, adolescent rats underwent bilateral surgical implantation of intracranial cannula in the mPFC (Experiment 6.1) or vHPC (Experiment 6.2). Rats in both the Pre and Alt-Pre groups were infused with PBS or MUSC 30 min prior to context preexposure in the CPFE.

### 6.2.3 Apparatus and Stimuli

The apparatus and stimuli used for the current experiments was as described in **Chapter 5**. The US was two 1.5mA foot-shocks separated by 1sec.

### 6.2.4 Behavioral Procedures and Drug Infusion

The CPFE procedures used in the current experiments was as described in **Chapter 5**. Briefly, rats were assigned to either the Pre or Alt-Pre group, and then

given PBS or MUSC 30 min prior to context preexposure in the CPFE. Rats in the behaviorally-naïve home-cage (HC) control group also received this infusion.

### 6.2.5 Brain Removal, Tissue Dissection, and qPCR

In Experiment 6.2, preexposed or behaviorally-naïve home-cage control rats rats given saline or muscimol were sacrificed 30 min after context exposure in the CPFE. Expression of the immediate early genes *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC, dHPC, and vHPC was analyzed via qPCR. The methods for rat sacrifice, brain removal, tissue dissection and qPCR are described in **Chapter 5.**

### 6.2.6 Histology

Cannula placements were verified in both the behavior and sacrificed groups as described in **Chapter 5**.

### 6.2.7 Data Analysis

#### 6.2.7.1 Analysis of Behavioral Data

The data were imported into STATISTICA 64 data analysis software and freezing behavior was analyzed as described in **Chapter 5**. Across both experiments, there were no main effects or interactions involving sex on freezing behavior (*p*s > .30), so the data were collapsed across this variable. Consistent with previous reports (Heroux et al., 2017; Robinson-Drummer et al., 2017), in both experiments, the Alt-Pre group was pooled across drug condition ("Pooled-Alt-Pre" group) as there was no significant difference between the two drug groups and they froze at uniformly low levels ($p > .25$). Post-shock and retention test freezing data were analyzed using 3 (Condition; Pooled-Alt-Pre, PBS, Muscimol) × 2 (within subjects; Phase of testing;

Post-shock vs. Retention) repeated measures ANOVA. Post-hoc contrasts were performed with Newman–Keuls tests. Consistent with previous foundational studies (Heroux et al., 2017, 2018; Robinson-Drummer et al., 2016, 2017), Rats were excluded from analysis as an outlier if they had a score of ± 1.96 standard deviations from the group mean (see below for the average Z-score of removed outliers).

#### 6.2.7.2 Analysis of IEG Expression

Relative gene expression for the IEGs *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC, dHPC, vHPC, and VMT was determined (see **Section 2.7**). The relative gene expression value was obtained by normalizing the data to the reference gene (*18s*) and to the home-cage control group average delta $C_T$ for each gene. Consistent with previous findings (Heroux et al., 2018; 2019), there were no main effects or interactions involving sex (ps > .20), so the data were collapsed across this variable in both experiments. There was also no difference between the raw data in HC group infused with PBS or muscimol, so the HC group was collapsed across drug condition (*p*s > .19) in both experiments. Gene expression was analyzed using a one-way ANOVA (HC, PBS, Muscimol) performed separately for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) brain region (mPFC, dHPC, vHPC, and VMT). Post-hoc contrasts were performed with Newman–Keuls tests. In Experiments 6.1 and 6.2, the number of outliers removed in each sampling condition can be found in **Tables 6.1 and 6.2**; the average Z-score of removed outliers was ± 3.57 (± 0.39 SEM) and ± 5.49 (± 0.82 SEM), respectively.

| Experiment 1: mPFC Inactivation during Context Preexposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Medial prefrontal cortex (mPFC) | | | | Dorsal hippocampus (dHPC) | | | |
| Genes | *F* | *p* | *n (HC, MUSC, PBS)* | *Outliers (HC, MUSC, PBS)* | *F* | *p* | *n (HC, MUSC, PBS)* | *Outliers (HC, MUSC, PBS)* |
| ***c-Fos*** | 15.52 | < .001 | 17, 11, 10 | 1, 1, 1 | 7.26 | < .003 | 17, 11, 9 | 1, 1, 1 |
| ***Arc*** | 17.42 | < .001 | 16, 11, 10 | 2, 1, 1 | 4.32 | < .024 | 18, 11, 9 | 0, 1, 1 |
| ***Egr-1*** | 5.43 | < .009 | 17, 11, 10 | 1, 1, 1 | 4.18 | < .024 | 16, 11, 10 | 2, 1, 0 |
| ***Npas4*** | 9.63 | < .001 | 17, 11, 10 | 1, 1, 1 | 8.47 | < .001 | 17, 11, 10 | 1, 1, 0 |
| | Ventral hippocampus (vHPC) | | | | Ventral midline thalamus (VMT) | | | |
| ***c-Fos*** | 8.77 | < .001 | 18, 11, 12 | 0, 1, 0 | 6.02 | < .008 | 13, 8, 8 | 1, 1, 0 |
| ***Arc*** | 9.85 | < .001 | 17, 12, 12 | 1, 0, 0 | 0.09 | > .90 | 12, 9, 8 | 2, 0, 0 |
| ***Egr-1*** | 0.22 | > .80 | 17, 12, 11 | 1, 0, 1 | 0.71 | > .50 | 13, 9, 8 | 1, 0, 0 |
| ***Npas4*** | 16.1 | < .001 | 17, 11, 11 | 1, 1, 1 | 1.41 | > .25 | 12, 7, 8 | 1, 2, 0 |

Table 6.1 Final group numbers (*n*), number of outliers removed (*HC*, *MUSC*, *PBS*), and statistical results for all factorial ANOVAs (see *F* and *p* values) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in each region (mPFC, dHPC, vHPC, VMT) for Experiment 6.1.

| Experiment 2: vHPC Inactivation during Context Preexposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Medial prefrontal cortex (mPFC) | | | | Dorsal hippocampus (dHPC) | | | |
| Genes | *F* | *p* | *n (HC, MUSC, PBS)* | *Outliers (HC, MUSC, PBS)* | *F* | *p* | *n (HC, MUSC, PBS)* | *Outliers (HC, MUSC, PBS)* |
| ***c-Fos*** | 12.2 | < .001 | 11, 10, 11 | 1, 2, 0 | 4.2 | < .05 | 9, 9, 10 | 1, 1, 1 |
| ***Arc*** | 17.16 | < .001 | 11, 11, 10 | 1, 1, 1 | 6.92 | < .01 | 9, 11, 10 | 1, 1, 1 |
| ***Egr-1*** | 3.82 | < .05 | 11, 11, 10 | 1, 1, 1 | 9.08 | < .001 | 9, 11, 10 | 1, 1, 1 |
| ***Npas4*** | 14.91 | < .001 | 11, 11, 9 | 1, 1, 2 | 11.64 | < .001 | 9, 11, 10 | 1, 1, 1 |
| | Ventral hippocampus (vHPC) | | | | Ventral midline thalamus (VMT) | | | |
| ***c-Fos*** | 9.81 | < .001 | 11, 11, 11 | 1, 1, 0 | 3.57 | < .05 | 11, 11, 12 | 1, 1, 0 |
| ***Arc*** | 7.91 | < .01 | 11, 11, 10 | 1, 1, 1 | 0.31 | > .70 | 11, 10, 10 | 1, 1, 2 |
| ***Egr-1*** | 0.8 | > .45 | 11, 11, 10 | 1, 1, 1 | 0.4 | > .67 | 12, 11, 11 | 0, 1, 1 |
| ***Npas4*** | 8.87 | < .001 | 11, 11, 10 | 1, 1, 1 | 1.64 | > .20 | 12, 11, 12 | 0, 1, 0 |

Table 6.2 Final group numbers (*n*), number of outliers removed (*HC*, *MUSC*, *PBS*), and statistical results for all factorial ANOVAs (see *F* and *p* values) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in each region (mPFC, dHPC, vHPC, VMT) for Experiment 6.2.

## 6.3 Results

### 6.3.1 Experiment 6.1: Prefrontal Inactivation during Context Exposure Impairs Contextual Fear Conditioning and IEG Expression in the mPFC, vHPC, and VMT

The purpose of Experiment 6.1 was to examine the effects of prefrontal inactivation during context learning on regional gene expression and contextual fear conditioning. Location of cannula tips is shown in **Figure 6.1** (left column). The experimental design and behavioral results can be seen in **Figure 6.2A-C.** We predicted that intra-mPFC muscimol infusion would abolish freezing and impair IEG expression in the mPFC, dHPC, and VMT. Behavioral analyses were run on 32 animals distributed across the following groups: Pooled-Alt-Pre (n=11), PBS-Pre (n=11), Muscimol-Pre (n=10). Repeated measures ANOVA revealed a significant main effect of Condition [$F(2, 26) = 44.53$, $p < .001$]. There was no main effect or any interactions involving Phase ($p$s > .23). The PBS-Pre group froze significantly more than any other group during both the post-shock and retention freezing tests ($p$s < .001). Additionally, there was no significant difference between Muscimol-Pre and the Pooled-Alt-Pre control group ($p$s > .38). These results indicate that prefrontal inactivation during context learning abolishes contextual fear conditioning during subsequent post-shock and retention test freezing phases of the CPFE.

Littermates of the behavior group were sacrificed 30 min after context exposure on the preexposure day of the CPFE. The IEG results can be seen in **Figure 6.3A-D**. Gene expression was analyzed using separate one-way ANOVAs (HC, PBS, and Muscimol) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) and region (mPFC, dHPC, vHPC, and VMT). Specific $F$ statistics, $p$ values, group $n$, and outliers removed for the sixteen one-way ANOVAs can be found in **Table 6.1**. Post hoc

contrasts revealed that, in the mPFC and vHPC, the Muscimol group had significantly reduced mRNA expression of *c-Fos*, *Arc*, and *Npas4* relative to the PBS group (*p*s < .007), with no difference between the Muscimol and HC control groups (*p*s > .15). For *Egr-1* expression, the same pattern was seen in prefrontal ($p < .01$) but not ventral hippocampal regions ($p > .80$). In the dHPC, while there was no difference between the PBS and Muscimol groups (*p*s >.30), these groups had significantly higher mRNA expression of every IEG above the HC group (*p*s < .05). Finally, in the VMT, the Muscimol group had significantly reduced mRNA expression of *c-Fos* relative to the PBS group ($p < .01$). No expression of *Arc*, *Egr-1*, or *Npas4* was detected above HC control levels in the VMT (*p*s > .25). Taken together, these results show that prefrontal inactivation during context learning impairs IEG expression in the mPFC, vHPC, and VMT.

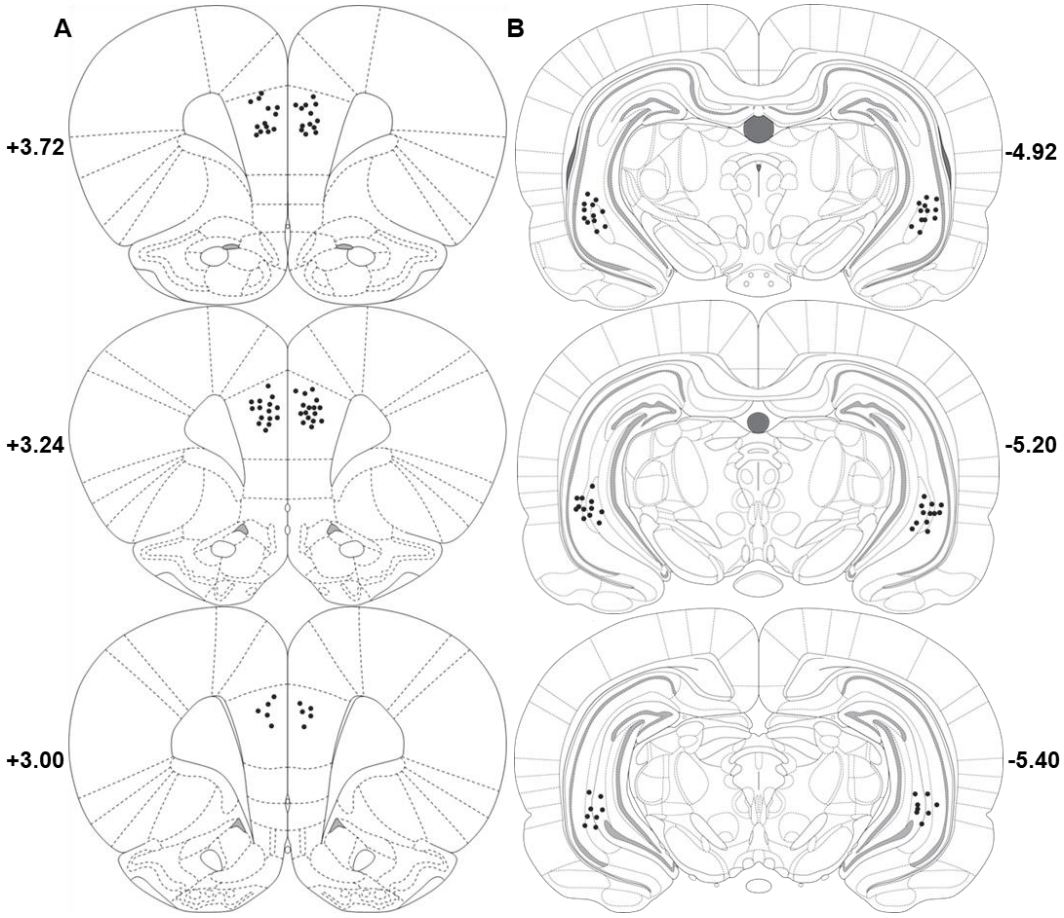


Figure 6.1 Schematic representation of the majority of injection cannula tip placements in the coronal plane for mPFC for Experiment 6.1 behavior (left panel, A) or in the vHPC for Experiment 6.2 behavior (right panel, B). Rats included in final analyses are represented by filled black dots. From *The Rat Brain in Stereotaxic Coordinates* (5th ed.), pp. 50, 52, 54, 180, 184, 188 by Paxinos & Watson, 2007, New York, NY: Academic Press. Copyright, 2005 by Elsevier Academic Press. Adapted (or reprinted) with permission.

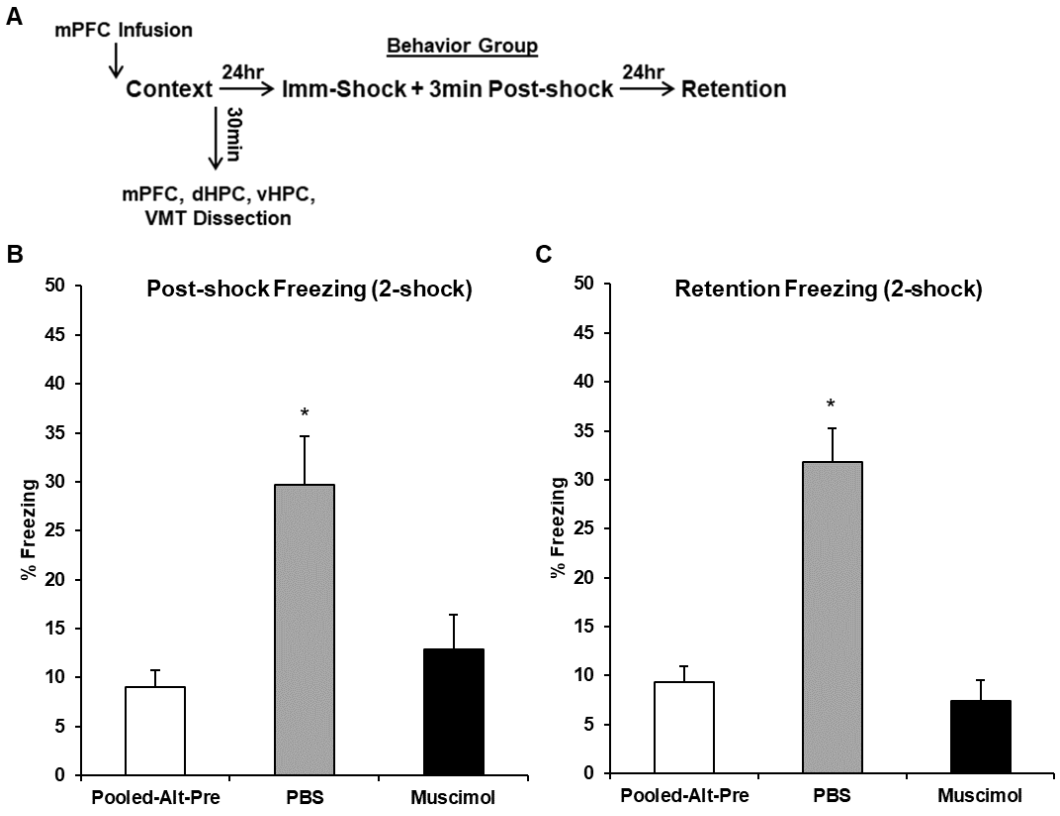


Figure 6.2 Behavioral design **(A)** and mean percent freezing (± SEM) for the 3-min post-shock **(B)** or 5-min retention **(C)** freezing tests in Experiment 6.1. **(A)** Rats were given intra-mPFC muscimol or PBS 30 min prior to context preexposure in the CPFE. **(B-C)** Muscimol-infused rats showed abolished post-shock and retention test freezing relative to PBS-infused rats ($p < .001$). There was no difference in freezing between the muscimol group and non-associative Alt-Pre control rats pooled across drug (Pooled-Alt-Pre, $ps > .38$). *****indicates $p < .001$

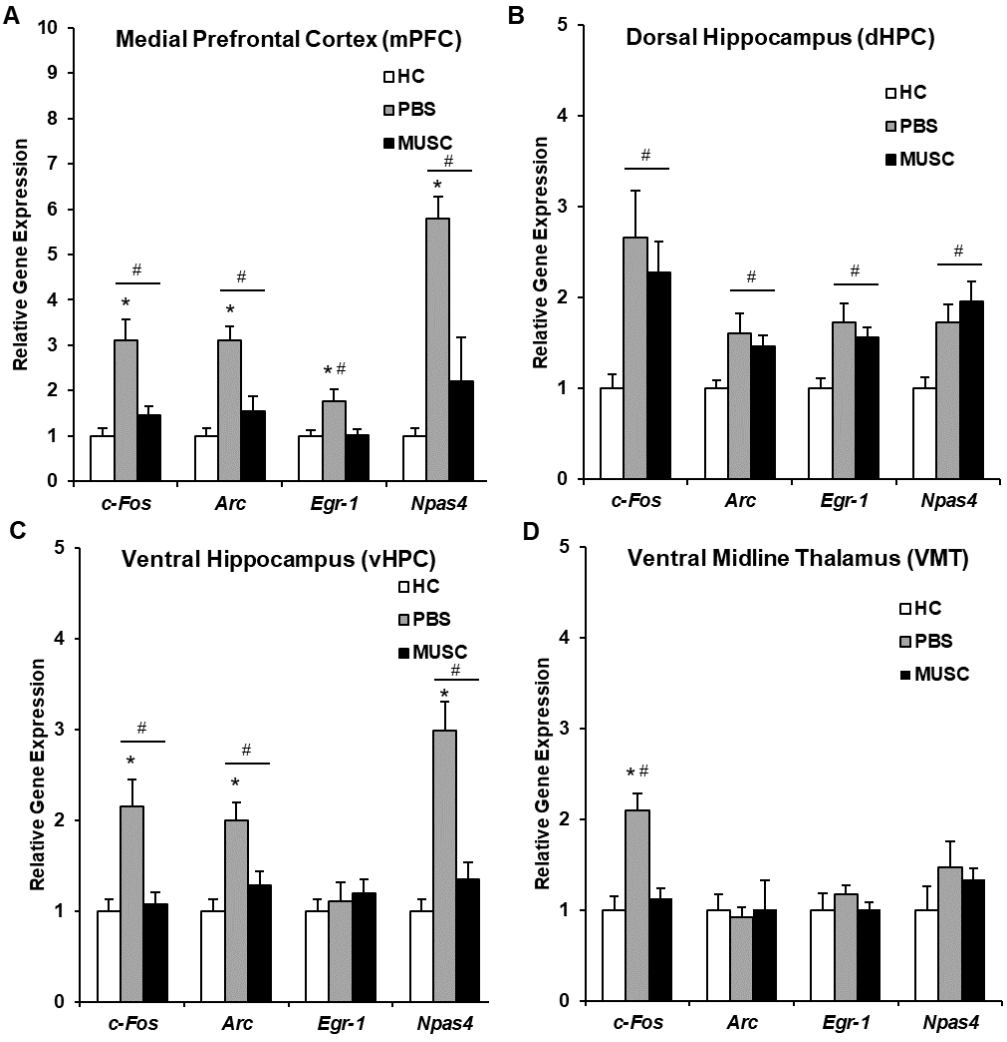


Figure 6.3 Relative mRNA expression (± SEM) of *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC **(A)**, dHPC **(B)**, vHPC **(C)**, and VMT **(D)** in rats infused with PBS, Muscimol, and behaviorally-naïve home-cage control rats (pooled across drug), sacrificed 30 min after context preexposure. **(A)** Muscimol infusion impaired expression of mPFC *c-Fos*, *Arc*, *Egr-1*, and *Npas4* relative to PBS-infused rats. **(B)** There was no difference between PBS and Muscimol groups for dHPC IEG expression, and both of these groups had elevated expression of all IEGs above the HC group **(C)** Muscimol infusion impaired expression of vHPC *c-Fos*, *Arc*, and *Npas4* relative to PBS-infused rats. **(D)** Muscimol infusion impaired expression of VMT *c-Fos* relative to PBS-infused rats, with no expression of any other IEG above HC. # indicates significant elevation above the HC group; * indicates a significant difference between PBS and Muscimol groups.

### 6.3.2 Experiment 6.2: Ventral Hippocampal Inactivation during Context Exposure Impairs Contextual Fear Conditioning and IEG Expression in the mPFC, dHPC, and vHPC

The purpose of Experiment 6.2 was to examine the effects of ventral hippocampal inactivation during context learning on regional gene expression and contextual fear conditioning. Location of cannula tips is shown in **Figure 6.1** (right column). The experimental design and behavioral results can be seen in **Figure 6.4A-C.** We predicted that intra-vHPC muscimol infusion would abolish freezing and impair IEG expression in the vHPC, VMT, and mPFC. Behavioral analyses were run on 30 animals distributed across the following groups: Pooled-Alt-Pre (n=10), PBS-Pre (n=10), Muscimol-Pre (n=10). Repeated measures ANOVA revealed a significant main effect of Condition [$F(2, 25) = 41.72$, $p < .001$]. Pooled-Alt-Pre and Muscimol-Pre rats failed to differ statistically ($p > .60$) and froze significantly less than PBS-Pre rats regardless of phase ($ps < .001$). In addition, there was a significant main effect of Phase of testing [$F(1, 25) = 6.00$, $p < .05$], with post-shock freezing being lower than retention test freezing. There were no interactions involving Phase ($p > .50$). Identical to Experiment 6.1, these results indicate that ventral hippocampal inactivation during context learning reduces subsequent post-shock and retention test freezing to the level of non-associative (Alt-Pre) controls during the CPFE.

The IEG results can be seen in **Figure 6.5A-D**. Specific *F* statistics, *p* values, group *n*, and outliers removed can be found in **Table 6.2** In the mPFC, dHPC, and vHPC, muscimol infusion reduced expression of every IEG to the level of behaviorally-naïve HC controls ($ps < .05$). The lack of an effect of muscimol on vHPC *Egr*-1 expression is one exception and likely reflects a floor effect as context preexposure did not elevate this expression above HC in either experiment. In the VMT, there was no difference between the PBS and Muscimol groups ($ps > .30$) for *c*-

*Fos* expression, and both of these groups showed higher *c-Fos* expression than the HC group (*p*s < .05). No expression of *Arc, Egr-1*, or *Npas4* was detected above HC control levels in the VMT (*p*s > .25). Taken together, these results show that ventral hippocampal inactivation during context learning impairs IEG expression in the mPFC, dHPC, and vHPC.

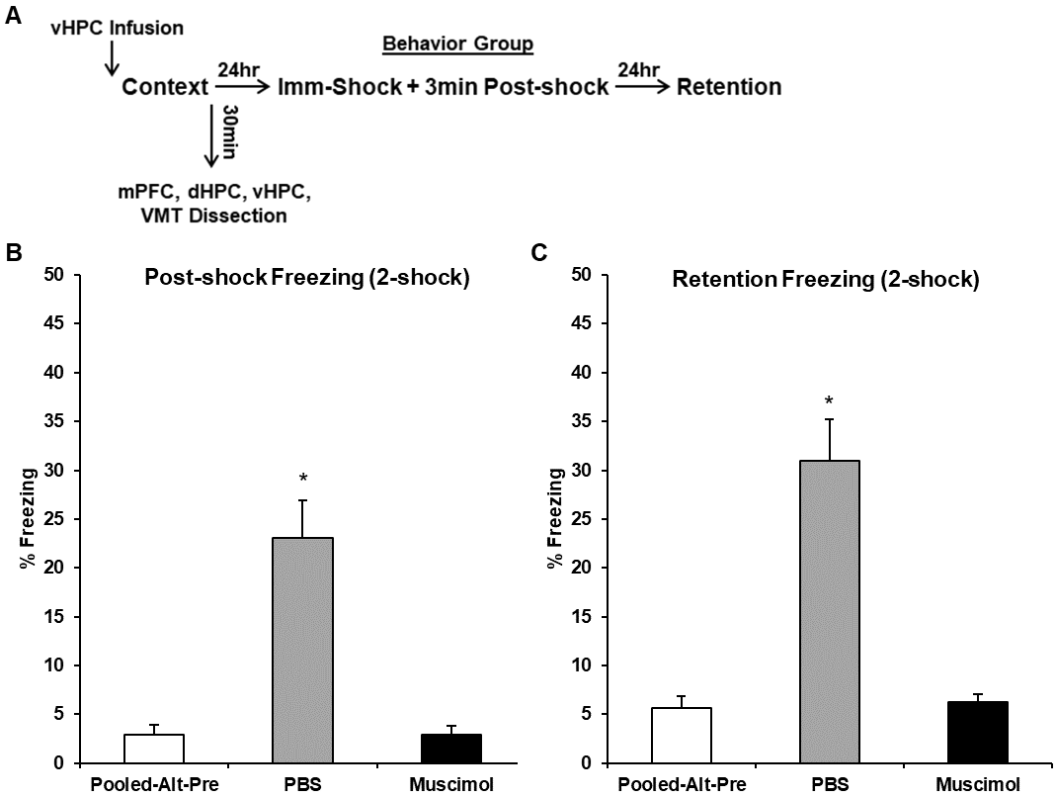


Figure 6.4 Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5 min retention **(C)** freezing tests in Experiment 6.2. **(A)** Rats were given intra-vHPC muscimol or PBS 30 min prior to context preexposure in the CPFE. **(B-C)** Muscimol-infused rats showed abolished post-shock and retention test freezing relative to PBS-infused rats ($p < .001$), with no difference between muscimol and Pooled-Alt-Pre groups ($ps > .60$). *****indicates $p < .001$

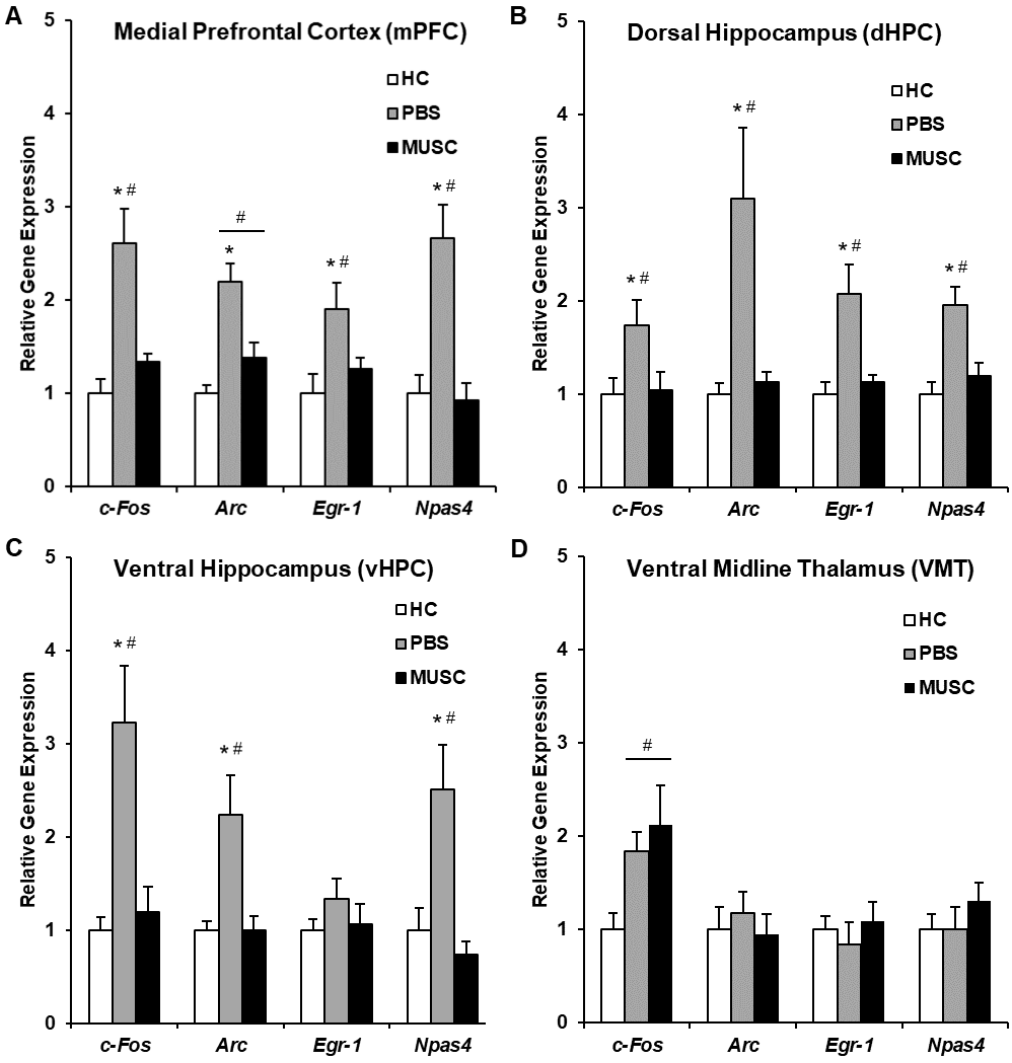


Figure 6.5 Relative mRNA expression (± SEM) of *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC **(A)**, dHPC **(B)**, vHPC **(C)**, and VMT **(D)** in rats infused with PBS, Muscimol, and behaviorally-naïve home-cage control rats (pooled across drug), sacrificed 30 min after context preexposure. **(A-C)** The muscimol group had impaired expression of *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC, dHPC, and vHPC relative to the PBS group. **(D)** Both the muscimol and PBS groups showed elevated expression of VMT *c-Fos* relative to the HC group, with no expression of any other IEG above HC. # indicates significant elevation above the HC group; * indicates a significant difference between PBS and Muscimol groups.

## 6.4 Discussion

The current study examined disruption of regional IEG expression caused by inactivation of mPFC (Experiment 6.1) or vHPC (Experiment 6.2) during incidental context learning. In both cases, inactivation abolished subsequent post-shock and retention test freezing in the CPFE. These results extend prior studies (Heroux et al., 2017; Rudy & Matus-Amat, 2005) by including a post-shock freezing test, which confirms that MUSC-infused rats either did not acquire or retain a context representation, or were unable to retrieve and/or associate it with foot-shock during immediate-shock training in the CPFE. In Experiment 6.1, mPFC inactivation attenuated expression of mPFC *c-Fos*, *Arc*, *Egr-1*, and *Npas4*, vHPC *c-Fos*, *Arc*, and *Npas4*, and VMT *c-Fos* to the level of behaviorally-naïve home-cage controls. There was no effect of prefrontal inactivation on dHPC IEG expression, suggesting that interactions between these two structures likely do not underlie long-term context memory in the CPFE. In Experiment 6.2, vHPC inactivation attenuated expression of all four IEGs in the mPFC, dHPC, and vHPC, with no effect on VMT IEG expression. Collectively, these results suggest that incidental context learning and/or memory processes are likely supported by extended mPFC-vHPC circuitry.

This suggestion of prefrontal and ventral hippocampal circuit involvement in these processes is subject to some important limitations. First, we cannot infer a functional role of regional IEG expression in supporting the CPFE without experiments involving intra-regional knockdown of specific IEGs or their downstream pathways (e.g., via ASO infusion or RNAi). Second, we cannot address the role of individual sub-regions or cell-specific expression as whole regions were dissected (e.g., mPFC consisted of the AC, PL, and IL). Third, as all tissue was collected at one time-point, it's possible that we missed the effects of muscimol infusion on regional

IEG expression occurring during later states of consolidation (e.g., intra-mPFC muscimol may disrupt dHPC IEG expression at later time-points). Importantly, one caveat that we *can* address is that the drug and behavioral effects seen in the current study cannot be attributed to "performance effects" of the muscimol infusion, such as impaired sensory processing (e.g., feature perception and encoding, shock sensitivity), motor performance (e.g., hyperactivity), or state dependency. Indeed, previous studies showed that mPFC or vHPC inactivation prior to sCFC has no effect on acquisition, and prior CPFE studies using cholinergic antagonists have ruled out state dependency effects following mPFC infusion (**Chapters 2 and 4;** Heroux et al., 2017; Robinson-Drummer et al., 2017; Rudy & Matus-Amat, 2005). In the following discussion, we consider the implications of our results in relation to previous research examining the roles of the mPFC, dHPC, vHPC, and VMT in distinct processes of CFC.

The current study further characterizes the involvement of the mPFC in incidental context learning and related CFC paradigms. Prefrontal inactivation during context preexposure abolished subsequent post-shock and retention test freezing, as well as experience-driven IEG expression in the mPFC, vHPC, and VMT (see Experiment 6.1). We've previously shown that prefrontal inactivation or muscarinic-receptor antagonism during any phase of the CPFE abolishes retention test freezing (**Chapter 4**; Heroux et al., 2017; Robinson-Drummer et al., 2017). This inactivation during training leaves post-shock freezing intact, indicating that the mPFC is not required for retrieving the context representation, associating it with shock, or the immediate expression of this association. In addition, rats can learn about contexts and acquire sCFC under prefrontal inactivation (Heroux et al., 2017), so the mPFC likely facilitates long-term consolidation of the conjunctive context representation following

context preexposure. This is supported by other fear conditioning research reviewed in **Chapter 2** suggesting that, in an intact rodent, the mPFC processes components of CFC in parallel with the dHPC (Corcoran & Quirk, 2007; Gilmartin et al., 2014; Gilmartin, Miyawaki, et al., 2013; Giustino & Maren, 2015; Heroux et al., 2017; Zelikowsky et al., 2013, 2014). Taken together, the data in this chapter support our previous point (**Chapter 2**) that strong recruitment of the prefrontal cortex in CFC occurs during procedures in which contexts are less salient (i.e., when competing discrete cues are present) or when there is a delay between learning the distinct components of CFC (i.e., in the CPFE; see Heroux et al., 2017).

We originally hypothesized that the mPFC interacts with dHPC via reciprocal connectivity with the VMT (i.e., rhomboid and nucleus reunions) to support context learning and memory (see Heroux et al., 2017). The VMT facilitates behaviorally-relevant communication and synchrony between these structures during spatial working memory tasks (Dolleman-van der Weel et al., 2019; Hallock et al., 2016; Maisson et al., 2018). In CFC procedures, mPFC-VMT and dHPC-VMT connectivity is important for encoding and contextual specificity of acquired context-shock associations (**Chapter 2;** Ramanathan et al., 2018; Xu & Südhof, 2013). Other evidence also suggests that mPFC and dHPC interact during context learning (Cholvin et al., 2017; Heroux et al., 2018, 2017; Heroux, Robinson-Drummer, et al., 2019; Hok et al., 2013, 2005; Kyd & Bilkey, 2003; Matus-Amat et al., 2004; Robinson-Drummer et al., 2016; Wiltgen et al., 2006; Zelikowsky et al., 2013, 2014). Despite these studies, the current results do ***not*** support this "mPFC-dHPC" interaction hypothesis. Indeed, prefrontal inactivation impaired IEG expression in the vHPC and VMT but *not* dHPC during context preexposure. These results suggest a possible role of mPFC-VMT-

vHPC connectivity in incidental context learning that demands further investigation with causal, pathway-specific manipulations.

The current chapter demonstrates a role of the vHPC, possibly in conjunction with the mPFC and dHPC, in incidental context learning and memory. Ventral hippocampal inactivation during context preexposure abolished both post-shock and retention test freezing, and disrupted IEG expression in the mPFC, dHPC, and vHPC in adolescent rats (see Experiment 6.2). This extends prior work demonstrating a necessary role of the vHPC during context preexposure in the CPFE in adult rats (Cullen et al., 2017; Rudy & Matus-Amat, 2005). Plasticity-related expression of phosphorylated ERK in the vHPC peaks around 2hr after context preexposure, and local anisomycin infusion during this consolidation period disrupts the CPFE (Cullen et al., 2017; Rudy & Matus-Amat, 2005). These CPFE studies challenge traditional views that the vHPC is not important for spatial learning, as assessed in Morris water maze and radial arm maze tasks (Bannerman et al., 1999; Fanselow & Dong, 2010; Feldon, Yee, Pothuizen, Zhang, & Jongen-Relo, 2004; E. I. Moser, Moser, & Andersen, 1993; M. B. Moser, Forrest, Moser, Morris, & Andersen, 1995). As discussed previously (**Chapter 2**), pre-training lesions or inactivation of the dHPC or vHPC leave CFC intact, while post-training manipulations produce severe retrograde amnesia (Ballesteros et al., 2014; Paul W. Frankland et al., 1998; Hunsaker & Kesner, 2008; J. Q. Lee et al., 2017; Maren et al., 1997; Maren & Holt, 2004; Matus-Amat et al., 2004; Wiltgen et al., 2006; Zelikowsky et al., 2013; W.-N. Zhang et al., 2014; Zhu et al., 2014). In contrast, pre-training NMDAR antagonism in the dHPC or vHPC disrupts contextual fear retention but leaves acquisition intact (Czerniawski et al., 2011; J. J. Kim et al., 1991, 1992; Quinn et al., 2005; Sanders & Fanselow, 2003;

Tayler et al., 2011). These studies suggest a similar role of both the dHPC and VHPC in contextual processes of CFC. In the current study, despite robust projections from the CA1 layer of vHPC to the VMT (Hoover & Vertes, 2012; Varela, Kumar, Yang, & Wilson, 2014), vHPC inactivation had no effect on VMT IEG expression. This suggests that vHPC inactivation impairs mPFC IEG expression by disrupting direct projections between these two structures. In contrast, the effects of vHPC inactivation on dHPC IEG expression are likely via indirect projections via the entorhinal cortex (Fanselow & Dong, 2010). This role of vHPC-mPFC connectivity in contextual and spatial learning is supported by recent studies manipulating these structures during CFC and spatial working memory tasks (Dolleman-van der Weel et al., 2019; W. Bin Kim & Cho, 2017). One recent study demonstrated that a subset of vHPC neurons projecting to the amygdala and mPFC preferentially respond to context exposure, and have excitatory influences on these structures during training (W. Bin Kim & Cho, 2017). In delay non-match to place and spatial conditional discrimination tasks, suppressing activity of vHPC terminals in the mPFC or activity of nucleus reunions during sample phases disrupts choice accuracy, indicating a role of these circuits in information related to spatial and/or working memory during these tasks (Hallock, Wang, Shaw, & Griffin, 2013; Maisson et al., 2018; Spellman et al., 2015). One mechanism of this impairment may be a disruption of theta synchrony and coupling between the mPFC-dHPC system as a result of altered vHPC activity (Dolleman-van der Weel et al., 2019; Spellman et al., 2015). Taken together, these studies suggest that vHPC-mPFC connectivity may underlie context memory and the effects seen in the current study. Furthermore, these results demonstrate a role of the vHPC in the

acquisition and/or consolidation of a context representation in the absence of aversive stimuli such as foot-shock.

These results may also inform the psychological and neural mechanisms underlying the ontogeny of distinct processes of the CPFE. Performance during post-shock and retention freezing tests in the CPFE emerges between PD17 and PD24 in rats (Jablonski et al., 2012; Schiffino et al., 2011). Until recently, intrinsic and experience-dependent development of the dHPC was thought to be the main neural substrate of the ontogeny of memory processes subserving the CPFE. This view should now be updated for several reasons. First, the CPFE requires activity in the mPFC, dHPC, vHPC, and BLA during context preexposure and immediate-shock training (Cullen et al., 2017; Heroux et al., 2017; Huff & Rudy, 2004; Matus-Amat et al., 2004; Miller et al., 2019; Robinson-Drummer et al., 2016; Rudy & Matus-Amat, 2005). Maturation of the amygdala cannot account for the ontogeny of CFC performance, as it can support acquisition and long-term memory of fear signaled by discrete cues such as odors or tones by PD17 (J. H. Kim, Li, Hamlin, McNally, & Richardson, 2012; Pugh & Rudy, 1996; Rudy & Morledge, 1994). Evidence of hippocampal and prefrontal involvement in supporting long-term memory in CFC emerges in tandem between PD17-24, depending on the sensory system the task requires (J. H. Kim et al., 2012; J. H. Kim & Richardson, 2007; Li, Kim, & Richardson, 2012; Travaglia, Bisaz, Sweet, Blitzer, & Alberini, 2016). There's little evidence to suggest a stronger role of hippocampal vs. prefrontal maturation in supporting the ontogeny of long-term memory in CFC. Indeed, while spatial firing properties of hippocampal place cells and entorhinal grid cells continue to develop over PD17-24, these cells do show location-specific firing patterns during context

exploration by PD17 (Langston et al., 2010; Wills, Barry, & Cacucci, 2012; Wills, Cacucci, Burgess, & O'Keefe, 2010). One potential caveat is that maturation of hippocampal replay in these cell ensembles during sleep may underlie development of successful long-term memory consolidation (Ghandour et al., 2019; Muessig, Lasek, Varsavsky, Cacucci, & Wills, 2019). In contrast, cytoarchitectonic and neurophysiological properties of the mPFC also develop over the first three weeks of life in the rat (van Eden & Uylings, 1985; Z. wei Zhang, 2004), the end of which corresponds to the period in which the mPFC starts contributing to CFC (J. H. Kim & Richardson, 2007; Li et al., 2012). Second, context preexposure induces robust activity- and plasticity-associated IEG expression in the mPFC that is absent in PD17 rats (Asok et al., 2013; Heroux et al., 2018; Robinson-Drummer et al., 2018; Schreiber et al., 2014). Finally, while PD17 rats fail to show 24hr retention test freezing in CFC, they are able to acquire a context-shock association and express this association in an immediate post-shock freezing test (Rudy & Morledge, 1994). Thus, the inability for PD17 rats to show the CPFE is likely not due to underdeveloped visual sensory processing or contextual feature encoding. One unique aspect of the CPFE is that it cannot be supported by individual features of the context, instead requiring the consolidation of these features into a conjunctive context representation to be retrieved and associated with shock during training (Jablonski et al., 2012; Rudy, 2009; Rudy & O'Reilly, 1999a). Therefore, the CPFE could require the mPFC and vHPC during context preexposure because these structures may facilitate successful consolidation of this conjunctive representation. Future experiments should use both gain-of-function and loss-of-function manipulations (e.g., pathway-specific optogenetic excitation and inhibition) in these structures across earlier development stages to provide important

insights into the neurobiology of learning and memory across the lifespan. In summary, development of molecular activity and connectivity between the mPFC, dHPC, and vHPC likely underlies the ontogeny the CPFE.

In summary, the current study provides novel evidence of medial prefrontal and ventral hippocampal circuitry being recruited for incidental contextual learning and memory in adolescent rats. Accordingly, severe disruption of activity- and plasticity-associated IEG expression in the mPFC and vHPC during context exposure was a common result across both experiments. While this hypothesis needs to be further directly tested via optogenetic or pharmacogenetic tools, our results suggest that mPFC inactivation impairs the CPFE by disrupting synchronization between the mPFC-VMT-HPC system, whereas vHPC inactivation impairs direct vHPC-mPFC and vHPC-dHPC communication engaged by incidental context exposure. Several basic predictions emerging from this hypothesis are discussed at the conclusion of this thesis (see **Section 9.3**, **Chapter 9).** Finally, this research further demonstrates the promise of the CPFE as a behavioral paradigm to explore interactions between prefrontal cortex and hippocampus during discrete processes of contextual fear conditioning.

# Chapter 7

# NEONATAL ETHANOL EXPOSURE IMPAIRS LONT-TERM CONTEXT MEMORY FORMATION AND PREFRONTAL IMMEDIATE EARLY GENE EXPRESSION IN ADOLESCENT RATS

## 7.1 Introduction

This chapter was recently published in *Behavioural Brain Research* (Heroux, Robinson-Drummer, et al., 2019), and the text appears here in adapted form. As reviewed in **Chapter 3**, neonatal alcohol exposure from PD4-9 results in a robust impairment in retention test freezing in the CPFE in adolescent and adult rats (G. F. Hamilton et al., 2011; Murawski et al., 2012; Murawski & Stanton, 2011). Despite this, these previous studies have been unable to elucidate whether this exposure impairs preexposure or training day processes as only retention test freezing was measured. Moreover, the role of the PFC in this disruption is not known. The purpose of the current study was to address these questions and to characterize the role of altered prefrontal molecular signaling in alcohol-induced disruption of the CPFE. Based on our recent findings demonstrating a necessary role of the mPFC in context memory (Heroux et al., 2017; Robinson-Drummer et al., 2017), we hypothesized that PD4-9 ethanol exposure would result in impaired context learning as well as disrupted prefrontal and hippocampal IEG expression on the preexposure day of the CPFE. Furthermore, we hypothesized that this disruption would result in reduced or abolished post-shock freezing in the CPFE, as alcohol-exposed rats will have encoded or

consolidated a weak context representation during context preexposure. While there is some evidence that *prenatal* alcohol exposure impairs prefrontal IEG expression (D. A. Hamilton, Akers, et al., 2010; Nagahara & Handa, 1995), to our knowledge, this is the one of the first studies to examine behaviorally-driven expression of multiple activity- and plasticity-associated IEGs in the mPFC after *neonatal* ethanol exposure in a rat model of FASD.

## 7.2 Materials and Methods

### 7.2.1 Subjects

Animal husbandry was as described as in **Chapter 5**. Across the three experiments, there were a total of 250 adolescent (PD31) Long Evans rats (131 females and 119 males), derived from 36 separate litters bred by the Office of Laboratory Animal Medicine at the University of Delaware.

### 7.2.2 Neonatal Alcohol Dosing

Neonatal ethanol dosing occurred over PD4-9 with methods described in **Chapter 5**. Briefly, rats were assigned to the EtOH or SI conditions. The EtOH group was given 5.25g/kg/d of alcohol in dosing milk over PD4-9, with blood for BAC analyses being collected via tail clip on PD4. The method for analyzing BACs was as described in **Chapter 5**.

### 7.2.3 Apparatus and Stimuli

The apparatus and stimuli used for the current experiments is as described in **Chapter 5** with one exception. Briefly, in Experiment 7.1A, one 1.5mA foot-shock

was used whereas two 1.5mA foot-shocks separated by 1sec were used in Experiments 7.1B, 7.2, and 7.3.

### 7.2.4 Behavioral Procedures

The CPFE (Experiments 7.1A-B and 7.3) and sCFC (Experiment 7.2) procedures used in the current experiments was as described in **Chapter 5**. Briefly, in Experiments 7.1A, 7.1B, and 7.3, SI and EtOH rats were assigned to the Pre or Alt-Pre conditions and underwent the CPFE using the multiple context preexposure procedure. In Experiment 7.2, SI and EtOH rats were assigned to the Delayed or Imm-shock conditions and underwent sCFC, with freezing being measured during a retention but not a post-shock freezing test.

### 7.2.5 Brain Removal, Tissue Dissection, and qPCR

In Experiment 7.3, rats in the SI-Pre, EtOH-Pre, and behaviorally naïve home-cage control group were sacrificed 30 min after context exposure in the CPFE. Expression of the immediate early genes *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC and dHPC was analyzed via qPCR as described in **Chapter 5**. Boundaries for tissue dissection can be seen in **Figure 5.1**.

### 7.2.6 Data Analysis

#### 7.2.6.1 Analysis of Neonatal and Adolescent Body Weight

Neonatal body weight was analyzed with a repeated measures ANOVA with a between-subjects factor of dosing condition (SI vs. EtOH) and the within-subjects factor of age (PD4 vs. PD9). Analyses of neonatal body weight was collapsed across both experiments, as there was no main effect or interaction as a function of

experiment (*p*s > .50). There were also no main effects or interactions involving sex in the PD4 and PD9 weights (*p*s > .40) so the data were collapsed across this variable at these ages. Body weight at PD31 was analyzed with a 2 (Sex; male vs. female) × 2 (Dosing condition; SI vs. EtOH) factorial ANOVA. Body weight averages (PD4, PD9, PD31 males and females) and BACs for both dosing conditions appear in **Table 7.1**.

| Experiment | Dose | n = | Body weight (Grams ± SEM) | | | | PD4 BACs (mg/dl) |
|---|---|---|---|---|---|---|---|
| | | | PD4 | PD9 | PD31 (males) | PD31 (females) | |
| **Experiment 7.1A** | **SI** | 30 | 10.89 ± 0.17 | 20.48 ± 0.35 | 111.25 ± 2.6 | 99.07 ± 0.92 | N/A |
| CPFE 1-shock | **EtOH** | 21 | 10.86 ± 0.19 | 17.54 ± 1.55* | 105.7 ± 2.45 | 97.64 ± 1.06 | 428.85 ± 10.21 |
| **Experiment 7.1B** | **SI** | 33 | 11.494 ± 0.2 | 20.75 ± 0.35 | 109.77 ± 2.68 | 100.3 ± 0.96 | N/A |
| CPFE 2-shock | **EtOH** | 33 | 11.34 ± 0.2 | 18.794 ± 0.34* | 110.8 ± 1.43 | 98.67 ± 1.03 | 417.48 ± 7.40 |
| **Experiment 7.2** | **SI** | 18 | 11.72 ± 0.26 | 21.08 ± 0.44 | 125.12 ± 2.8 | 110.7 ± 2.12 | N/A |
| sCFC | **EtOH** | 15 | 11.85 ± 0.23 | 17.44 ± 0.52* | 118.1 ± 3.34 | 108.44 ± 2.17 | 407.05 ± 7.04 |
| **Experiment 7.3** | **SI** | 55 | 10.92 ± 0.13 | 20.18 ± 0.33 | 112.07 ± 1.78 | 99.43 ± 0.70 | N/A |
| CPFE Pre day IEG | **EtOH** | 45 | 11.24 ± 0.18 | 17.74 ± 0.25* | 108.86 ± 2.04 | 98.35 ± 1.05 | 423.11 ± 7.45 |
| **Collapsed** | **SI** | 137 | 11.16 ± 0.09 | 20.50 ± 0.19 | 113.03 ± 1.29 | 101.17 ± 0.07 | N/A |
| **Expts. 7.1-7.3** | **EtOH** | 115 | 11.29 ± 0.11 | 17.89 ± 0.17* | 110.1 ± 1.41 | 99.80 ± 1.61 | 420.31 ± 4.69 |

Table 7.1 Body weights and BACs for Experiments 7.1A, 7.1B, 7.2, and 7.3. Average body weights (in grams ± SE) are given from the SI and EtOH groups at the first and last day of the dosing period (PD4 and PD9, respectively) and the first day of behavioral training (PD31). BACs (in mg/dl ± SE) were taken from blood samples collected on PD4 from the EtOH group. * indicates a significant difference between the SI and EtOH groups.

#### 7.2.6.2 Analysis of Behavioral Data

Scoring and behavioral data analysis is as described in **Chapter 5**. There were no main effects or interactions involving sex on freezing behavior across any of the experiments (*p*s > .20), so the data were collapsed across this variable. In Experiments 7.1 and 7.3, post-shock and retention test freezing data were analyzed using 2 (Dosing condition; SI vs. EtOH) × 2 (Exposure condition; Pre vs. Alt-Pre) × 2 (within subjects; Phase of testing; Post-shock vs. Retention) repeated measures ANOVAs. In Experiment 7.2, the immediate shock control group was pooled across dosing condition as there was no significant difference between the SI and EtOH groups and they froze uniformly low ($p > .50$). Retention test freezing data were analyzed using a one-way ANOVA (EtOH-Delayed vs. SI-Delayed vs. Pooled-Imm-Shock). Post-hoc contrasts were performed with Newman–Keuls tests. Rats were excluded from analysis as an outlier if they had a score of ± 1.96 standard deviations from the group mean, however, the average Z-score of removed outliers averaged across all experiments was ± 5.45 (± 1.04 SEM). One animal from each group (EtOH-Alt-Pre, EtOH-Pre, SI-Alt-Pre, and SI-Pre in Experiments 7.1 and 7.3; SI-Delayed and EtOH-Delayed in Experiment 7.2) was excluded as an outlier from both the post-shock and retention freezing data in each experiment.

#### 7.2.6.3 Analysis of IEG Expression

Relative gene expression for the IEGs *c-Fos, Arc, Egr-1*, and *Npas4* in the mPFC and dHPC was determined in Experiment 7.3 as described in **Chapter 5**. Consistent with previous findings (Heroux et al., 2018), there were no main effects or interactions involving sex across any of the experiments (*p*s > .30), so the data were collapsed across this variable. There was also no difference between the raw data in

HC group dosed with alcohol or sham-intubated, so the HC group was collapsed across dosing condition (*p*s > .20). Gene expression in Experiment 7.3 was analyzed using a one-way ANOVA (HC, SI-Pre, and EtOH-Pre) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in both the mPFC and dHPC. Post-hoc contrasts were performed with Newman–Keuls tests. The number of outliers removed in each sampling condition in Experiment 7.3 can be found in **Table 7.2**. Two rats were excluded from IEG analyses in Experiment 7.3 because of insufficient RNA for cDNA synthesis. The average Z-score of removed outliers was ± 3.22 (± 0.22 SEM).

| Experiment 3: CPFE Preexposure Day IEG Expression (Pre Group) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Medial prefrontal cortex (mPFC) | | | | Dorsal hippocampus (dHPC) | | | |
| Genes | *F* | *p* | *n (HC, EtOH, SI)* | *Outliers (HC, EtOH, SI)* | *F* | *p* | *n (HC, EtOH, SI)* | *Outliers (HC, EtOH, SI)* |
| ***c-Fos*** | 73.81 | < .0001 | 20, 10, 11 | 2, 1, 1 | 25.32 | < .0001 | 20, 11, 10 | 2, 1, 1 |
| ***Arc*** | 45.36 | < .0001 | 19, 10, 11 | 2, 1, 1 | 20.75 | < .0001 | 20, 11, 10 | 2, 1, 1 |
| ***Egr-1*** | 6.62 | < .001 | 20, 10, 11 | 2, 1, 1 | 1.14 | > .30 | 20, 11, 10 | 2, 1, 1 |
| ***Npas4*** | 33.7 | < .0001 | 21, 10, 11 | 1, 1, 1 | 19.87 | < .0001 | 20, 11, 11 | 2, 1, 0 |

Table 7.2 Final group numbers (*n*), number of outliers removed (*HC, Alt-Pre, Pre*), and statistical results for all one-way ANOVAs (see *F* and *p* values) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in each region (mPFC and dHPC) for Experiment 7.3.

## 7.3 Results

### 7.3.1 Body Weight and BACs

Body weight averages for sham-intubated and alcohol-exposed rats at PD4, PD9, and PD31 appear in **Table 7.1**. Both the SI and EtOH groups gained substantial weight during the dosing period (PD4-PD9) up until the age of testing (PD31). A 2 (Dosing condition; SI vs. EtOH) × 2 (Age; PD4 vs. PD9) repeated measures ANOVA revealed significant main effects of Dosing condition [$F(1, 250) = 45.10$, $p < .001$], Age [$F(1, 250) = 5372.73$, $p < .001$], as well as a Dosing condition × Age interaction [$F(1, 250) = 156.90$, $p < .001$]. Newman-Keuls tests revealed no difference between group weights on PD4 ($p$s > .50), but on PD9, EtOH rats weighed about 13% less than SI rats ($p$s < .001). Transient growth retardation in ethanol treated rats over this dosing period has been reported previously (Brown et al., 2007; G. F. Hamilton et al., 2011; Murawski et al., 2012; Murawski & Stanton, 2010). Ethanol did not alter body weight at the time of testing. A 2 (Dosing condition; SI vs. EtOH) × 2 (Sex; male vs. female) factorial ANOVA performed on PD31 body weights revealed a significant main effect of Sex [$F(1, 248) = 111.45$, $p < .001$] but not Dosing condition [$F(1, 248) = 3.86$, $p > .05$], with no interaction between these two variables [$F(1, 248) = 0.41$, $p > .50$]. Females had reduced body weights compared to males at PD31 regardless of dosing condition (see **Table 7.1**).

BACs taken from the blood samples of the EtOH group on PD4 are also shown in **Table 7.1** (grouped by experiment and then collapsed across all experiments). The EtOH group showed an average BAC of 422.45 ± 4.69 mg/dl. There was no significant effect of experiment (7.1A vs. 7.1B vs. 7.2 vs. 7.3) or sex (male vs. female) on BACs ($p$s > .30).

### 7.3.2 Experiment 7.1A: PD4-9 Alcohol Exposure Abolishes Post-shock and Retention Test Freezing Under 1-Shock Reinforcement

The purpose of Experiment 1A was to examine the effects of PD4-9 ethanol exposure on post-shock and retention test freezing in the CPFE. The behavioral design and results for Experiment 1A can be seen in **Figure 7.1**. Analyses for Experiment 7.1A were run on 47 rats distributed across the following groups: EtOH-Alt-Pre (n=7), EtOH-Pre (n=12), SI-Alt-Pre (n=13), and SI-Pre (n=15). Repeated measures ANOVA revealed a significant main effect of Dosing [$F(1, 41) = 55.35$, $p < .001$], Exposure [$F(1, 41) = 45.92$, $p < .001$], and a significant Dosing × Exposure interaction [$F(1, 41) = 26.98$, $p < .001$]. There was no main effect or any interactions involving Phase ($ps > .08$). The SI-Pre group froze significantly more than all other groups during both the post-shock and retention freezing tests ($ps < .001$). There was no difference between EtOH rats preexposed to the training context (EtOH-Pre) and non-associative controls preexposed to an alternate context (EtOH-Alt-Pre or SI-Alt-Pre; $ps > .50$) in either phase. These results show that PD4-9 ethanol exposure abolishes post-shock and retention test freezing in the CPFE.

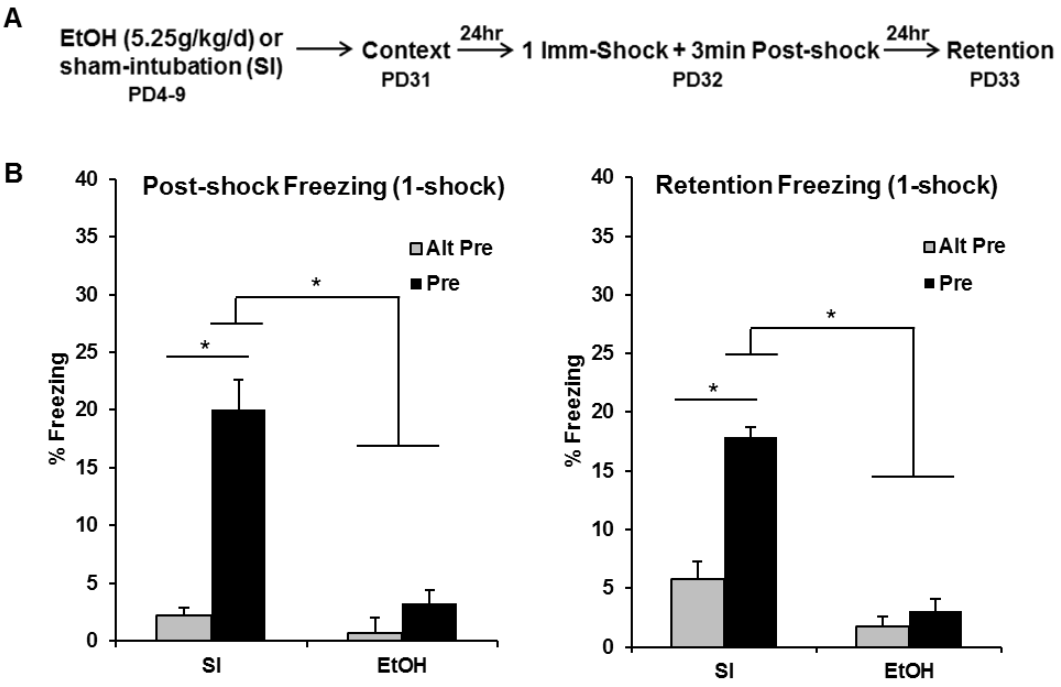


Figure 7.1 Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5 min retention **(C)** freezing tests. **(A)** Rats were given alcohol or sham-intubation from PD4-9, and then run through the full three-day CPFE procedure from PD31-33. The US was *one* immediate shock. **(B, C)** The SI-Pre group froze significantly higher than the EtOH-Pre group and both Alt-Pre control groups during the 3min post-shock and 5 min retention freezing tests (*p*s < .001). ***** indicates $p < .001$

### 7.3.3 Experiment 7.1B: PD4-9 Alcohol Exposure Impairs Post-shock and Retention Test Freezing Under 2-Shock Reinforcement

The purpose of Experiment 7.1B was to determine whether or not increasing the strength of the immediate-shock reinforcement (i.e., by increasing number of shocks to 2 instead of 1) would alter behavioral impairments seen in Experiment 7.1A. The behavioral design and results for Experiment 7.1B can be seen in **Figure 7.2**. Analyses for Experiment 7.1B were run on 61 rats distributed across the following groups: EtOH-Alt-Pre (n=9), EtOH-Pre (n=21), SI-Alt-Pre (n=11), and SI-Pre (n=20). Repeated measures ANOVA revealed a significant main effect of Dosing [$F(1, 55) = 6.99$, $p < .01$], Exposure [$F(1, 55) = 34.04$, $p < .001$], and a significant Dosing × Exposure interaction [$F(1, 55) = 4.97$, $p < .05$]. There was no main effect or any interactions involving the repeated measure of Phase (*ps* > .15). SI-Pre rats froze significantly more than EtOH-Pre rats during the post-shock ($p < .05$) and retention ($p < .001$) freezing tests. While there was no difference between EtOH rats and both Alt-Pre groups in retention freezing ($p > .30$), EtOH rats froze significantly more than the Alt-Pre groups during the post-shock freezing test ($p < .05$). These results suggest that doubling the amount of shock-reinforcement given during training improves post-shock freezing in EtOH rats but is not fully effective in rescuing ethanol-induced impairment of the CPFE during either test phase.

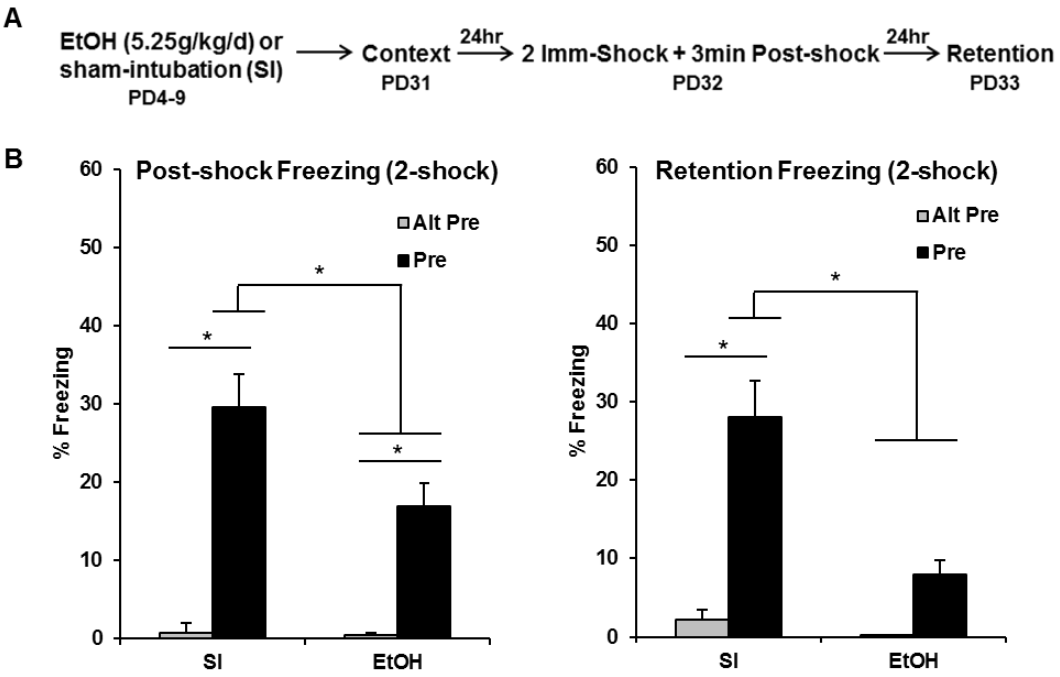


Figure 7.2 Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5 min retention **(C)** freezing tests. **(A)** Rats were given alcohol or sham-intubation from PD4-9, and then run through the full three-day CPFE procedure from PD31-33. The US was *two* immediate shocks. **(B)** Rats in the SI-Pre group froze significantly higher than the EtOH-Pre group ($p < .05$), which froze significantly higher than both Alt-Pre groups during the post-shock freezing test ($p < .05$). **(C)** The SI-Pre group froze significantly higher than every other group during the retention freezing test ($ps < .001$), with no difference between the other groups ($ps > .20$). ***** indicates $p < .05$

### 7.3.4 Experiment 7.2: PD4-9 Alcohol Exposure Does Not Impair Retention Test Freezing in sCFC

The purpose of Experiment 7.2 was to examine whether or not ethanol-exposed rats are impaired in standard contextual fear conditioning, in which learning about the context and acquiring a context-shock association occurs within the same trial. The behavioral design and results for Experiment 7.2 can be seen in **Figure 7.3**. Analyses for Experiment 7.2 were run on 33 rats distributed across the following groups: EtOH-Delayed (n=10), SI-Delayed (n=11), and Pooled-Imm-Shock (n=12; SI=6, EtOH=6). One-way ANOVA revealed a significant main effect of Group [$F(1, 30) = 25.47$, $p < .001$]. Both SI-Delayed and EtOH-Delayed groups froze significantly higher than Pooled-Imm-Shock control group ($p$s $< .001$), with no difference between the two Delayed groups ($p > .50$). These results show that ethanol-exposed rats are able to acquire and retain contextual fear when context exposure and foot-shock occur within the same trial.

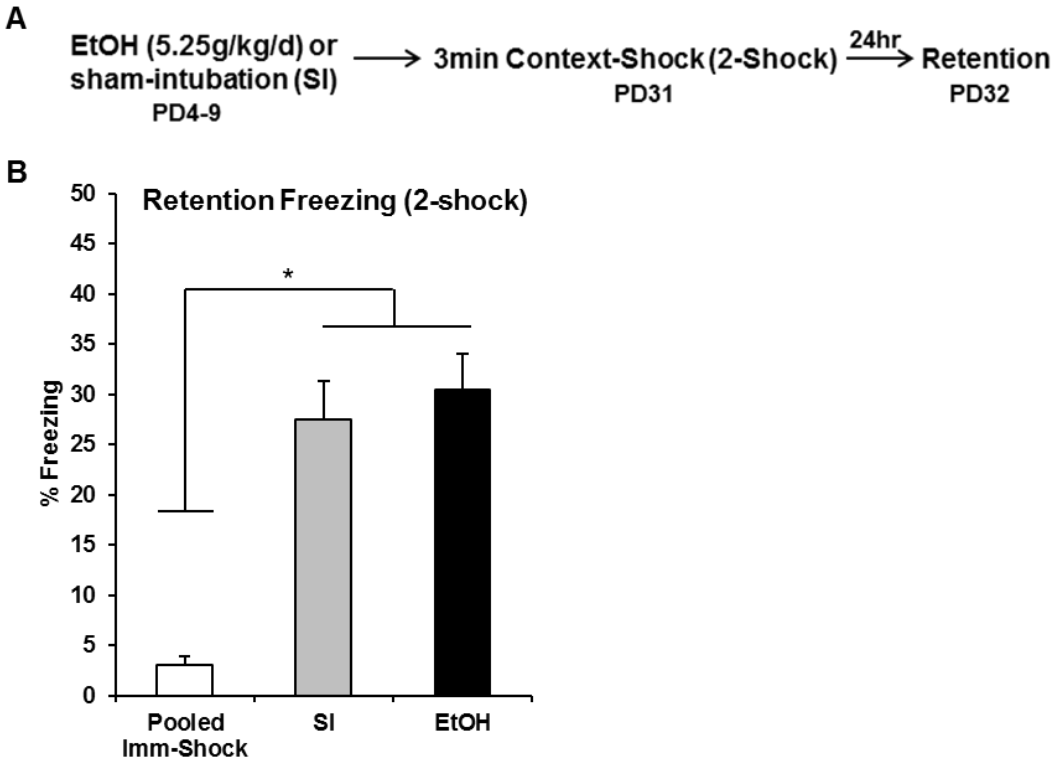


Figure 7.3 Behavioral design **(A)** and mean percent freezing (± SEM) for the 5 min retention **(B)** freezing test occurring 24hrs after context-shock pairing. **(A)** Rats were given alcohol or sham-intubation from PD4-9, and then run through the two-day sCFC procedure from PD31-32. The US was two foot-shocks occurring three minutes after chamber entry. **(B)** There was no difference in retention test freezing between the SI-Delayed and the EtOH-Delayed groups (*p*s > .58), with both groups freezing significantly higher than an immediate-shock control group collapsed across dosing condition (*p*s < .001). ***** indicates $p < .05$

### 7.3.5 Experiment 7.3: PD4-9 Alcohol Exposure Impairs Medial-Prefrontal but Not Dorsal-Hippocampal IEG Expression during Context Exposure

The purpose of Experiment 7.3 was to determine whether impaired context memory in ethanol-exposed rats is accompanied by disrupted IEG expression in the mPFC and dHPC during context preexposure (see **Figure 5.1** for dissected regions). The behavioral design and results for Experiment 7.3 can be seen in **Figure 7.4A-C**. Analyses for Experiment 7.3 were run on 48 rats distributed across the following groups: EtOH-Alt-Pre (n=8), EtOH-Pre (n=13), SI-Alt-Pre (n=11), and SI-Pre (n=16). Repeated measures ANOVA revealed a significant main effect of Dosing [$F(1, 42) = 22.15$, $p < .001$], Exposure [$F(1, 42) = 34.98$, $p < .001$], and a significant Dosing × Exposure interaction [$F(1, 42) = 7.88$, $p < .01$]. There was no main effect or any interactions involving Phase (*ps* > .40). The SI-Pre group froze significantly more than any other group during both the post-shock and retention freezing tests (*p*s < .001). Additionally, there was no significant difference between EtOH-Pre and the Alt-Pre control groups (*ps* > .20).

The IEG results can be seen in **Figure 7.4D-E**. Gene expression in Experiment 3 was analyzed using a one-way ANOVA (HC, SI-Pre, and EtOH-Pre) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in both the mPFC and dHPC. Specific *F* statistics, *p* values, group *n*, and outliers removed for all eight one-way ANOVAs for Experiment 3 can be found in **Table 7.2**. Post hoc contrasts revealed that, in the mPFC, EtOH rats showed significantly reduced mRNA expression of every IEG (*c-Fos, Arc*, *Egr-1*, and *Npas4*; see **Figure 7.4D**) compared to SI rats (*p*s < .001). However, expression of *c-Fos, Arc*, and *Npas4* in EtOH rats was still significantly above HC control levels (*p*s > .01). This disruption of IEG expression in the EtOH group was not seen in the dHPC, with both SI and EtOH rats having significantly higher expression of *c-Fos, Arc*, and

*Npas4* above HC control levels, with no difference between the two dosing groups (*p*s > .18; see **Figure 7.4E**). These results indicate that neonatal PD4-9 ethanol exposure impairs prefrontal but not hippocampal IEG expression induced by context exposure.

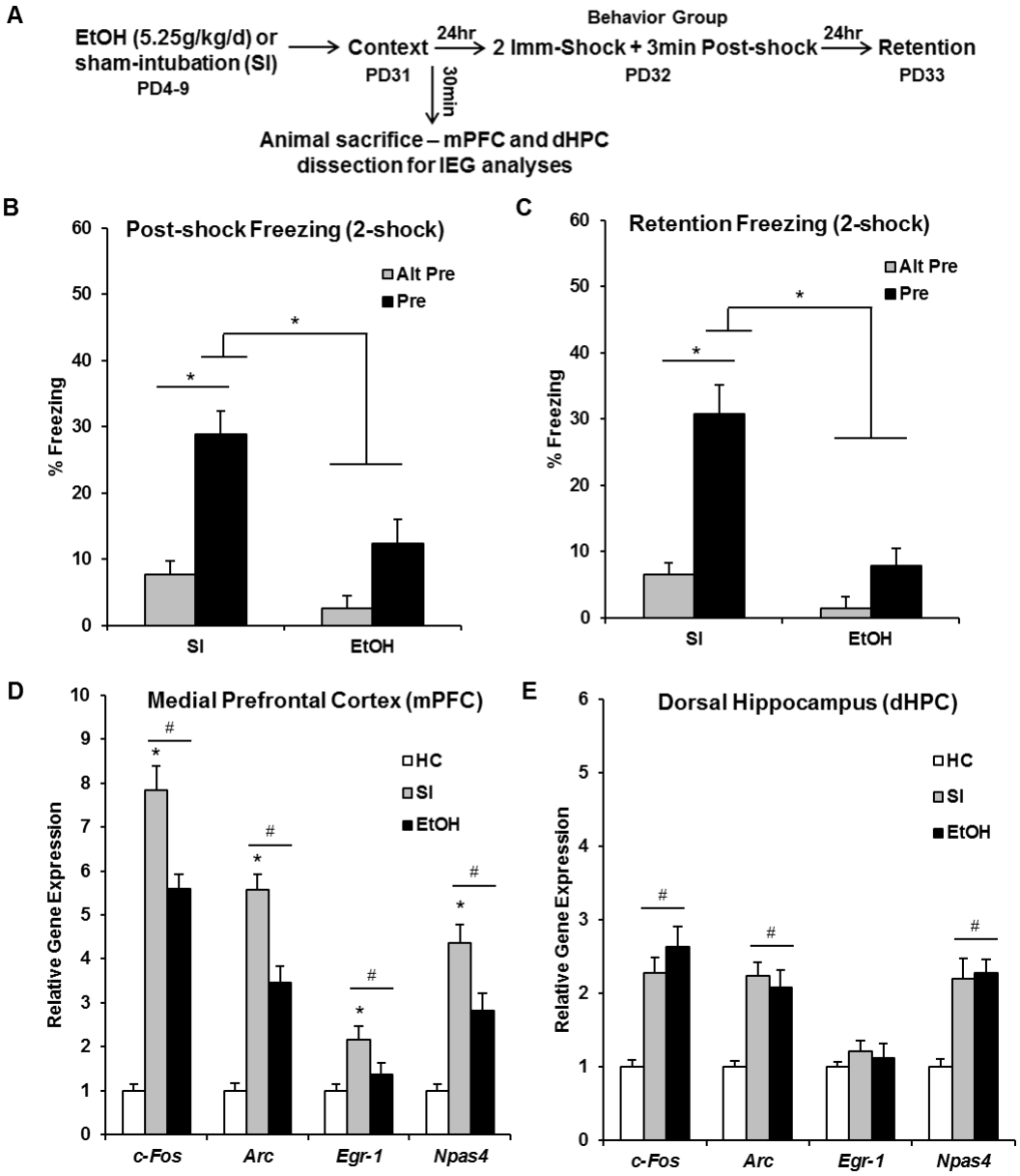
A
EtOH (5.25g/kg/d) or sham-intubation (SI)
PD4-9
Context
PD31
24hr
30min
Behavior Group
2 Imm-Shock + 3min Post-shock
PD32
24hr
Retention
PD33
Animal sacrifice – mPFC and dHPC dissection for IEG analyses
B
Post-shock Freezing (2-shock)
Alt Pre
Pre
% Freezing
SI
EtOH
C
Retention Freezing (2-shock)
D
Medial Prefrontal Cortex (mPFC)
HC
SI
EtOH
Relative Gene Expression
c-Fos
Arc
Egr-1
Npas4
E
Dorsal Hippocampus (dHPC)

Figure 7.4 Behavioral design **(A)** and data **(B, C)**, and post-context-preexposure IEG expression in the mPFC **(D)** and dHPC (**E**) for the HC, EtOH, and SI experimental groups. **(A)** Rats were given alcohol or sham-intubation from PD4-9, and then run through the full three-day CPFE procedure from PD31-33. Littermates of this behavior group were sacrificed 30 min after context exposure and IEG mRNA expression in the mPFC and dHPC was assayed via qPCR. **(B, C)** The SI-Pre group froze significantly higher than the EtOH-Pre group and both Alt-Pre control groups during the 3min post-shock and 5 min retention freezing tests (*p*s < .001). **(D)** The SI group had significantly higher expression of every IEG above both the EtOH and baseline HC control group (*p*s > .001). The EtOH group had significantly higher *c-Fos*, *Arc*, and *Npas4* expression than the HC group (*p*s > .001). **(E)** Both SI and EtOH groups had significantly higher expression of c-Fos, Arc, and Npas4 above HC control levels, with no difference between the two dosing groups (ps > .18). **#** indicates significant elevation above the HC group; ***** indicates a significant difference between SI and EtOH groups.

## 7.4 Discussion

The current set of experiments examined the disruption caused by neonatal alcohol exposure on context and contextual fear learning in the CPFE in adolescent rats. Consistent with previous CPFE studies (Goodfellow & Lindquist, 2014; G. F. Hamilton et al., 2011; Murawski et al., 2012; Murawski & Stanton, 2010, 2011), high binge-like doses of ethanol given over PD4-9 abolished 24-hr retention test freezing (Experiments 7.1A, 7.1B, and 7.3). Importantly, previous research has been unable to elucidate whether this disruption in retention reflects an impairment in preexposure or training day processes. In the current study, ethanol exposure left freezing in sCFC intact (Experiment 7.2), but post-shock freezing on the training day of the CPFE was severely impaired in ethanol-exposed rats regardless of reinforcement intensity (i.e., one vs. two shocks) used (Experiments 7.1A, 7.1B, and 7.3). Furthermore, ethanol-exposed rats showed a selective disruption in medial prefrontal but not dorsal hippocampal expression of the IEGs *Arc*, *c-Fos*, *Egr-1*, and *Npas4* induced by context preexposure in the CPFE (Experiment 7.3**)**. Taken together, these results indicate that PD4-9 ethanol exposure disrupts prefrontal but not hippocampal activity- and plasticity-associated gene expression during incidental context learning, which may reflect a disruption in configural memory processes of the CPFE (i.e., acquisition, consolidation, or retrieval of a conjunctive context representation).

Extending previous work examining retention only (Goodfellow & Lindquist, 2014; G. F. Hamilton et al., 2011; Murawski et al., 2012; Murawski & Stanton, 2010, 2011), PD4-9 ethanol exposure abolished post-shock and retention test freezing in the CPFE. In contrast, retention freezing in single-trial sCFC in ethanol-exposed rats was spared. Accordingly, ethanol-induced disruptions in the CPFE cannot be attributed to reduced shock sensitivity, hyperactivity, or impaired context exploration or feature

perception. Shortening the interval between context exposure and immediate-shock training to 2hr rescues 24hr retention test freezing in ethanol-exposed rats, suggesting that alcohol exposure does not impair the ability to associate a previously learned context with a shock in the CPFE (Goodfellow & Lindquist, 2014). Therefore, the observed ethanol-induced deficit in post-shock freezing likely reflects a disruption in the consolidation of the conjunctive context representation after context preexposure in the CPFE. Additionally, because the CPFE requires the mPFC and dHPC during all three phases (Heroux et al., 2017; Matus-Amat et al., 2004; Robinson-Drummer et al., 2016, 2017), whereas single-trial conditioning in sCFC depends on the dHPC but not mPFC (Heroux et al., 2017; Wiltgen et al., 2006), these results implicate impaired prefrontal mechanisms of the CPFE. This notion is consistent with the observed ethanol-induced disruptions in prefrontal but not hippocampal IEG expression during context learning (Experiment 7.3).

Our lab has previously characterized disruptions in brain and behavior after different exposure windows in the rat, notably after PD4-6, PD4-9, and PD7-9. Unlike in the PD4-9 or PD7-9 dosing scenarios, PD4-6 ethanol exposure has no effect on retention test freezing in the CPFE (Murawski & Stanton, 2011). While this might suggest that the disruptive effects of ethanol exposure could be solely attributed to the PD7-9 window, this exposure leaves post-shock freezing on the training day of the CPFE intact (Jablonski & Stanton, 2014). Moreover, impaired contextual fear retention in these rats is associated with reduced prefrontal *Egr-1* mRNA expression on the *training day* of the CPFE (Jablonski et al., 2018). In contrast, we report that the broader PD4-9 exposure results in impaired context memory and prefrontal IEG expression on the *preexposure day* of the CPFE. Our lab has previously shown that

PD4-9 ethanol exposure results in a knockdown of hippocampal c-Fos protein expression and CA1 pyramidal cell loss on the preexposure day (Murawski et al., 2012). The current study does not replicate this ethanol-induced knockdown in hippocampal *c-Fos* expression. These different outcomes could reflect procedural differences, i.e., sampling entire dHPC vs. CA1, sampling mRNA vs. protein, one vs. two daily doses, and different amounts of context exposure. Despite this, these results suggest that mechanisms accounting for the more severe behavioral impairment after PD4-9 ethanol exposure likely extend beyond disruptions in hippocampal neuroanatomy and function.

The current results significantly expand upon previous literature demonstrating that developmental ethanol exposure alters prefrontal neuroanatomy and function. Ethanol exposure from PD2-6 or PD4-9 results in decreased dendritic complexity and branching in layer II/III pyramidal neurons (Granato et al., 2003; G. F. Hamilton et al., 2010). Concurrent with reduced dendritic complexity, this exposure also alters voltage-gated $Ca^{2+}$ channel activity while decreasing dendritic spiking number and duration in layer V pyramidal neurons in the prefrontal cortex (Granato et al., 2012). While studies of neonatal exposure are limited, *prenatal* ethanol exposure alters experience-dependent gene expression in the prefrontal cortex. For example, ethanol exposure throughout gestation results in a decrease in the expression of the IEGs *Arc* and *c-Fos* in the prelimbic cortex (PL) in adult rats during wrestling and social interaction behavioral tasks (D. A. Hamilton, Akers, et al., 2010; D. A. Hamilton, Candelaria-Cook, et al., 2010). A narrower second-trimester equivalent exposure disrupts the expression of the transcription factors *c-Fos* and *jun-B* in the PL and anterior cingulate during testing in a T-maze alternation task (Nagahara & Handa,

1995). Finally, late gestational exposure also results in decreased *c-Fos* protein expression in the infralimbic cortex during an open field task in adolescent rats (Fabio et al., 2013). Although the current study failed to find any significant changes in hippocampal gene expression or activity during contextual fear conditioning in ethanol-exposed rats, our findings do not discount previous research demonstrating robust ethanol-induced neuroanatomical and molecular dysfunction in the hippocampus (see **Chapter 3** for discussion). Notably, alcohol exposure causes lasting disruptions in hippocampal pyramidal cell counts, muscarinic receptor signaling, protein expression, and increases repressive epigenetic markers via increased DNA methylation (Livy et al., 2003; Monk et al., 2012; Murawski et al., 2012; Otero et al., 2012). We implicate targeting of cognitive processes depending on prefrontal circuitry that have historically been misattributed solely due to hippocampal dysfunction, especially in incidental contextual learning and memory that is critical for the CPFE.

In summary, our findings demonstrate that PD4-9 ethanol exposure impairs the acquisition and/or consolidation of context memory, resulting in abolished post-shock and retention test freezing in the CPFE. This behavioral deficit was associated with a robust impairment in immediate early gene expression in the medial prefrontal cortex of ethanol-exposed rats during the preexposure day of the CPFE. Finally, ethanol-exposed rats were unimpaired during a "prefrontal-independent" but "hippocampal-dependent" sCFC protocol (Heroux et al., 2017; Wiltgen et al., 2006), which furthers highlights prefrontal targeting and rules out any "performance effects" of alcohol exposure on behavior. It is important to note that the current findings may be limited to the developmental period of behavioral observation (i.e., in adolescent rats), so more research is needed on the impact of developmental alcohol exposure on behavior

across the lifespan. Nevertheless, these findings are important because prefrontal dysfunction is an integral hallmark of FASD in humans, but animal models have thus far largely failed to capture prefrontal dysfunction after third-trimester equivalent exposure. The CPFE has proven to be a promising behavioral paradigm that can facilitate linking alterations in prefrontal and hippocampal function to discrete phases of learning and memory that are impaired by developmental alcohol exposure in rats. More research is needed to establish a link between disrupted brain circuitry and cognitive dysfunction in animal models of FASD.

# Chapter 8

# CHOLINERGIC RESCUE OF NEUROCOGNITIVE INSULT FOLLOWING THIRD-TRIMESTER EQUIVALENT ALCOHOL EXPOSURE IN RATS

## 8.1 Introduction

This chapter was recently published in *Neurobiology of Learning and Memory* (Heroux, Horgan, Rosen, et al., 2019), and the text appears here in adapted form. As reviewed in **Chapter 3 and 4**, our lab has shown that systemic administration of the acetylcholinesterase inhibitor PHY prior to every phase of the CPFE rescues retention test freezing in PD7-9 alcohol-exposed rats (Dokovna et al., 2013). The specific phase of the CPFE that mediates this rescue effect, the regional neural correlates of the effect, and whether it would extend to the more severe PD4-9 exposure window is not known. Therefore, the purpose of the current study was to examine the effects of augmenting cholinergic signaling on behavioral performance and mPFC, dHPC, and vHPC IEG expression in PD4-9 ethanol-exposed rats. In addition, the current study analyzed vHPC IEG expression given our discovery of mPFC-vHPC circuitry being involved during context preexposure in the CPFE (see **Chapter 6**). In Experiment 8.1, we extended our earlier findings with PD7-9 exposure (Dokovna et al., 2013) by examining whether or not systemic administration of PHY prior to all three phases would rescue freezing in rats receiving PD4-9 ethanol exposure. In Experiment 8.2, we sought to determine if this same injection prior to just context learning would rescue disrupted behavior, and more importantly, prefrontal IEG expression previously observed during the CPFE (**Chapter 7**; Heroux, Robinson-Drummer, et al.,

2019; Jablonski et al., 2018). To our knowledge, this is the first study to examine the effects of boosting cholinergic function (via PHY) on neural activity during behavior in rats receiving neonatal ethanol exposure during the brain growth spurt.

## 8.2 Materials and Methods

### 8.2.1 Subjects

Animal husbandry was as described as in **Chapter 5**. Across both experiments, there were a total of 197 adolescent (PD31) Long Evans rats (97 females and 100 males), derived from 30 separate litters bred by the Office of Laboratory Animal Medicine at the University of Delaware.

### 8.2.2 Neonatal Alcohol Dosing

Dosing procedures (SI and EtOH groups), window (PD4-9), dose (5.25g/kg/d), blood collection, and BAC analyses occurred as described in **Chapters 5 and 7**.

### 8.2.3 Apparatus and Stimuli

The apparatus and stimuli used for the current experiments were as described in **Chapter 5**. The US was two 1.5mA foot-shocks separated by 1sec.

### 8.2.4 Behavioral Procedures and Drug Injection

The multiple preexposure CPFE procedure used in the current experiments was as described in **Chapter 5, 6, and 7**. Briefly, SI and EtOH rats were assigned to the Pre condition (Experiment 8.1) or the Pre and Alt-Pre conditions (Experiment 8.2 and underwent the CPFE. The Alt-Pre condition was omitted from Experiment 8.1 to streamline the design and to avoid needless duplication with Experiment 8.2 where inclusion of the Alt-Pre group to analyze freezing behavior was more useful. Rats in

both dosing groups received systemic intraperitoneal (i.p.) injections of 1ml/kg of 0.01mg/ml/kg physostigmine or the vehicle sterile saline. This injection occurred 30 min prior to all three phases in Experiment 8.1, and 30 min prior to only context preexposure in Experiment 8.2. Post-shock (3 min) and retention test (5 min) freezing was measured in both experiments. In Experiment 8.2, behavior was assessed in littermates of rats assigned to IEG assays.

### 8.2.5 Brain Removal, Tissue Dissection, and qPCR

In Experiment 8.2, rats in the SI-Pre-PHY, SI-Pre-SAL, EtOH-Pre-PHY, EtOH-Pre-SAL, and home-cage control group were sacrificed 30 min after context exposure in the CPFE. Expression of the immediate early genes *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC, dHPC, and vHPC was analyzed via qPCR as described in **Chapter 5** and was identical to **Chapters 6 and 7**.

### 8.2.6 Data Analysis

#### 8.2.6.1 Analysis of Neonatal and Adolescent Body Weight

Neonatal body weight was analyzed with repeated measures ANOVA with a between-subjects factor of dosing condition (SI vs. EtOH) and the within-subjects factor of age (PD4 vs. PD9). There were no main effects or interactions involving sex in the PD4 and PD9 weights (ps > .05) so the data were collapsed across this variable at these ages. Body weight at PD31 was analyzed with a 2 (Sex; male vs. female) × 2 (Dosing condition; SI vs. EtOH) factorial ANOVA.

#### 8.2.6.2 Analysis of Behavioral Data

Scoring and behavioral data analysis is as described in **Chapter 5**. There were no main effects or interactions involving sex on freezing behavior (*p*s > .05), so the data were collapsed across this variable. In Experiment 8.1, freezing data were analyzed using 2 (Dosing condition; SI vs. EtOH) × 2 (Drug condition; SAL vs. PHY) × 2 (within subjects; phase of testing; Post-shock vs. Retention) repeated measures ANOVA. In Experiment 8.2 the Alt-Pre group was pooled across drug but not dosing condition (Pooled-Alt-Pre group) as there was no significant difference between the two drug groups and they froze at uniformly low levels ($p > .34$). Therefore, freezing data were analyzed using 2 (Dosing; SI vs. EtOH) × 3 (Condition; SAL vs. PHY vs. Pooled-Alt-Pre) × 2 (within subjects; phase of testing; Post-shock vs. Retention) repeated measures ANOVA. Post-hoc contrasts were performed with Newman–Keuls tests. Rats were excluded from analysis as an outlier as in previous experiments, and the Z-score of removed outliers averaged  3.91 (± 0.97 SEM). Outliers removed were as follows: Experiment 1 [(Post-shock: SI-PHY=1, SI-SAL=1)] and Experiment 2 [(Post-shock: EtOH-Pooled-Alt-Pre=1, EtOH-PHY=1, EtOH-SAL=1, SI-PHY=1, SI-SAL=1; Retention: EtOH-Pooled-Alt-Pre=1, SI-Pooled-Alt-Pre=1, EtOH-PHY=2, EtOH-SAL=2)].

#### 8.2.6.3 Analysis of IEG Expression

Relative gene expression for the IEGs *c-Fos, Arc, Egr-1*, and *Npas4* in the mPFC, dHPC, and vHPC was determined. The relative gene expression value was obtained by normalizing the data to the reference gene (*18s*) and to the average delta $C_T$ of the home-cage control group for each gene. Consistent with previous experiments, lack of statistical effects (ps > .30) led to pooling data across sex. There

was also no difference between the raw data in HC group injected with SAL or PHY, so the HC group was collapsed across drug condition (*p*s > .35). Gene expression was analyzed using a 2 (Dosing condition; SI vs. EtOH) × 2 (Drug condition; SAL vs. PHY) factorial ANOVA for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in the mPFC, dHPC, and vHPC. Post-hoc contrasts were performed with Newman–Keuls tests and a Dunnett's test that contrasted the four experimental groups with the HC control group. The average Z-score of removed outliers was ± 3.14 (± 0.12 SEM; see **Table 8.2**).

## 8.3 Results

### 8.3.1 Body Weight and BACs

Body weight averages for sham-intubated and alcohol-exposed rats at PD4 and PD9 appear in **Table 8.1**. Both the SI and EtOH groups gained substantial weight during the dosing period (PD4-PD9) up until the age of testing (PD31). A 2 (Dosing; SI vs. EtOH) × 2 (Age; PD4 vs. PD9) repeated measures ANOVA revealed significant main effects of Dosing condition [$F(1, 195) = 67.59$, $p < .001$], Age [$F(1, 195) = 7213.30$, $p < .001$], as well as a Dosing × Age interaction [$F(1, 195) = 316.35$, $p < .001$]. This reflected no treatment effect on PD4 weights ($p > .70$), but on PD9, EtOH rats weighed about 15% less than SI rats (*p*s < .001). Ethanol did not alter body weight at the time of behavioral testing (**Table 8.1)**. Females had lower body weights compared to males at PD31 regardless of dosing condition [$F(1, 193) = 56.33$, $p < .001$]. Finally, the average BAC value taken from the blood samples of the EtOH group in each experiment can be seen in **Table 8.1**.

| Experiment | Dose | n = | Body weight (Grams ± SEM) | | | | PD4 BACs (mg/dl) |
|---|---|---|---|---|---|---|---|
| | | | PD4 | PD9 | PD31 (males) | PD31 (females) | |
| Experiment 8.1 | SI | 23 | 12.1 ± 0.27 | 20.8 ± 0.36 | 115.42 ± 2.57 | 108.55 ± 2.87 | N/A |
| | EtOH | 20 | 12.06 ± 0.26 | 17.68 ± 0.46* | 110.55 ± 3.24 | 101.27 ± 1.69 | 389.33 ± 4.78 |
| Experiment 8.2 | SI | 77 | 11.49 ± 0.14 | 21.23 ± 0.21 | 116.17 ± 1.46 | 106.16 ± 1.57 | N/A |
| | EtOH | 77 | 11.42 ± 0.15 | 17.79 ± 0.20* | 115 ± 1.21 | 105.76 ± 1.39 | 407.80 ± 2.86 |
| Experiment 8.1 and 8.2 | SI | 100 | 11.63 ± 0.16 | 21.13 ± 0.18 | 116 ± 1.26 | 106.70 ± 1.37 | N/A |
| Collapsed | EtOH | 97 | 11.56 ± 0.14 | 17.77 ± 0.19* | 114.17 ± 1.16 | 104.75 ± 1.16 | 403.99 ± 2.58 |

Table 8.1 Body weights and BACs for Experiment 8.1 and 8.2. Average body weights (in grams ± SE) are given from the SI and EtOH groups at the first and last day of the dosing period (PD4 and PD9, respectively) and the first day of behavioral training (PD31). BACs (in mg/dl ± SE) were taken from blood samples collected on PD4 from the EtOH group. * indicates a significant difference between the SI and EtOH groups.

### 8.3.2 Experiment 8.1: Systemic Administration of Physostigmine Prior to Every Phase Rescues the CPFE in Alcohol-exposed Rats

The purpose of Experiment 8.1 was to determine whether impaired post-shock and retention test freezing in ethanol-exposed rats could be rescued by systemic administration of PHY prior to each phase of the CPFE. The behavioral procedure and results for Experiment 8.1 can be seen in **Figure 8.1A-C**. A 2 (Dosing: EtOH vs. SI) x 2 (Drug: SAL vs. PHY) x Phase (Post-shock vs. Retention) between-within factorial design assessed rats exposed only to Context A (Pre, see Section 8.2.4). We predicted that alcohol would impair freezing in SAL- but not PHY-treated rats.

Analyses for Experiment 8.1 were run on 41 rats distributed across the following groups: SI-SAL-Pre (n=11), SI-PHY-Pre (n=11), EtOH-SAL-Pre (n=9), and EtOH-PHY-PRE (n=10). ANOVA revealed a significant main effect of Dosing [$F(1, 35) = 23.91$, $p < .001$], Drug [$F(1, 35) = 8.40$, $p < .01$], and a significant Dosing × Drug interaction [$F(1, 35) = 18.23$, $p < .001$]. There was no main effect or any interactions involving the repeated measure of Phase (*ps* > .45). EtOH-SAL rats showed abolished post-shock and retention test freezing relative to SI-SAL rats ($p < .01$). PHY treatment eliminated this effect by restoring freezing in the EtOH-PHY group to control levels. Importantly, PHY did not alter freezing levels of SI rats. It also didn't change freezing during the preexposure session which was uniformly low across experimental conditions; SI and EtOH animals freezing levels were 2.53 ± 0.54 SEM and 1.41 ± 0.21 SEM, respectively. These results demonstrate that systemic PHY administration prior to all three phases of the CPFE rescues impaired context conditioning in ethanol-exposed rats.

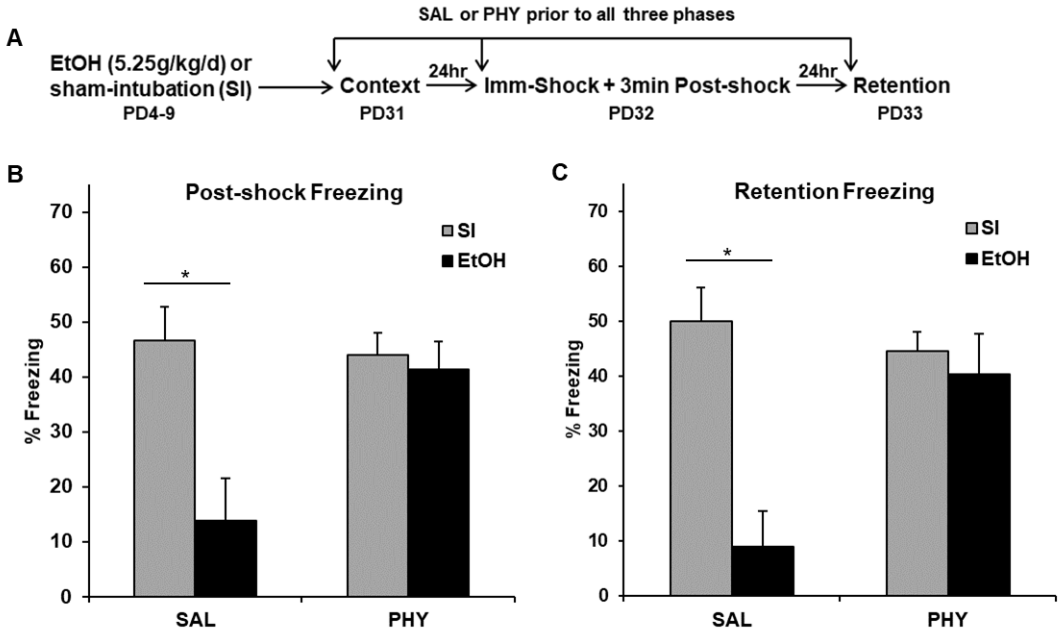


Figure 8.1 Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5 min retention **(C)** freezing tests in Experiment 8.1. **(A)** Rats were given alcohol (EtOH) or sham-intubation (SI) from PD4-9, and given physostigmine (PHY) or saline (SAL) prior to each phase of the CPFE procedure occurring from PD31-33. **(B-C)** SAL-treated EtOH group rats showed abolished post-shock and retention test freezing compared to SI rats regardless of drug treatment. PHY treatment prior to each phase of the CPFE restored freezing in EtOH rats compared to their SAL-treated counterparts. ***** indicates $p < .05$

### 8.3.3 Experiment 8.2: Systemic Administration of Physostigmine Prior to Context Preexposure Rescues the CPFE and Elevates IEG Expression in Alcohol-exposed Rats

#### 8.3.3.1 Behavioral Results

The purpose of Experiment 8.2 was to examine the effects of physostigmine given only prior to context preexposure on IEG expression and impaired freezing in ethanol-exposed rats. The behavioral procedure and results for Experiment 8.2 can be seen in **Figure 8.2A-C**. Freezing behavior was assessed with a 2 (Dosing: EtOH vs. SI) x 3 (Condition: SAL vs. PHY vs. Pooled-Alt-Pre) x 2 (Phase of testing: Post-shock vs. Retention) between-within factorial design. We predicted that alcohol would impair freezing in SAL- but not PHY-treated rats, with no difference in freezing between EtOH-SAL rats and the non-associative Alt-Pre control group pooled across drug (Pooled-Alt-Pre).

Analyses for Experiment 8.2 were run on 75 rats distributed across the following groups: SI-Pooled-Alt-Pre (n=13), SI-SAL-Pre (n=13), SI-PHY-Pre (n=12), EtOH-Pooled-Alt-Pre (n=12), EtOH-SAL-Pre (n=12), and EtOH-PHY-PRE (n=13). ANOVA revealed a significant main effect of Dosing [$F(1, 63) = 16.58$, $p < .001$], Condition [$F(2, 63) = 64.56$, $p < .001$], and a significant Dosing × Condition interaction [$F(2, 63) = 21.82$, $p < .001$]. There was no main effect or any interactions involving the repeated measure of Phase ($ps > .05$). Freezing above the non-associative Pooled-Alt-Pre control group was present in SI rats regardless of drug, but was only present in EtOH rats given PHY ($ps < .01$). Consistent with Experiment 8.1, SI and EtOH freezing levels during the preexposure were 2.91 ± 0.75 SEM and 3.23 ± 0.51 SEM, respectively. These results demonstrate that PHY treatment prior to context learning rescues abolished freezing in the CPFE.

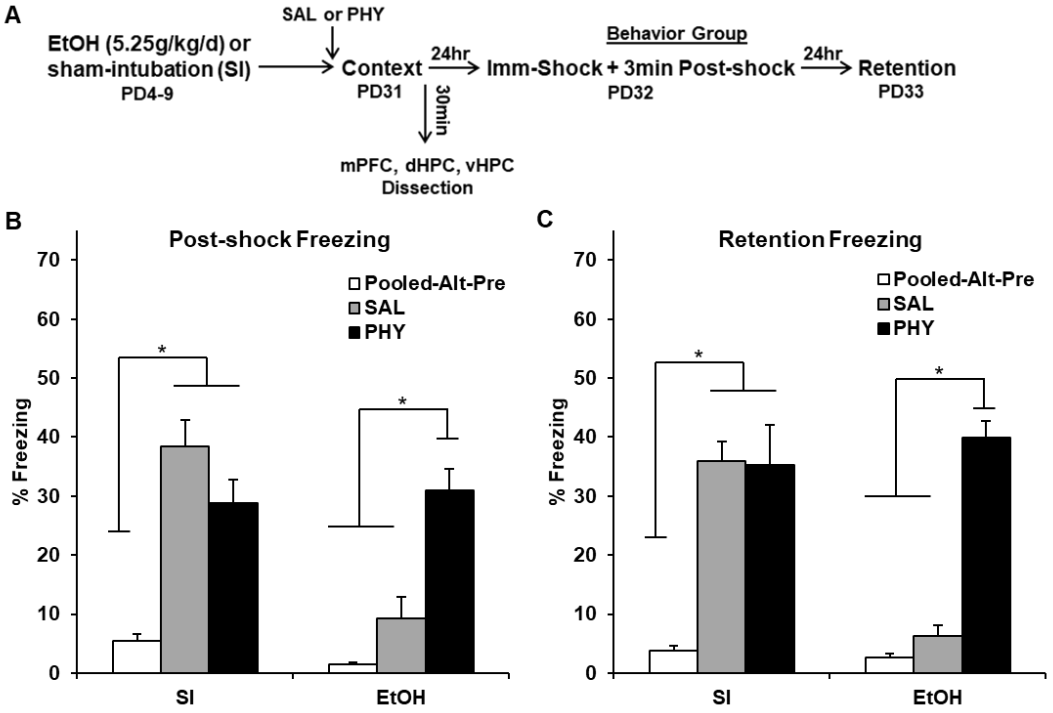


Figure 8.2 Behavioral design **(A)** and mean percent freezing (± SEM) for the 3min post-shock **(B)** or 5 min retention **(C)** freezing tests in Experiment 8.2. **(A)** Rats were given alcohol (EtOH) or sham-intubation (SI) from PD4-9, and given physostigmine (PHY) only prior to context preexposure in the CPFE. **(B-C)** Freezing above the non-associative Pooled-Alt-Pre control group was present in SI rats regardless of drug, but was only present in EtOH rats given PHY but not SAL. ***** indicates $p < .05$

#### 8.3.3.2 IEG Results

Littermates of the behavior group were sacrificed 30 min after context exposure on the preexposure day of the CPFE. The IEG results can be seen in **Figure 8.3A-C**. The statistical results, group sizes, and number of outliers removed in each sampling condition can be found in **Table 8.2**.

In the mPFC (**Fig. 8.3A**), the EtOH-SAL group showed significantly reduced mRNA expression of the IEGs *c-Fos, Arc,* and *Npas4* compared to Group SI-SAL ($p < .001$), replicating Experiment 7.2 (**Chapter 7**). Interestingly, PHY administration in EtOH rats specifically rescued prefrontal expression of *c-Fos* (i.e., the increase occurred in EtOH-PHY but not SI-PHY rats; $p$s $< .01$). Both SI and EtOH rats given PHY showed significantly elevated expression of the IEGs *Arc* and *Npas4* above their respective saline-treated groups ($p$s $< .01$), indicating that PHY enhanced overall expression but failed to reverse ethanol effects on expression of these IEGs. While PHY treatment did raise prefrontal *egr-1* expression in SI rats ($p < .05$), there was no difference in expression between the EtOH groups ($p$s $> .20$). Dunnett's tests revealed that every group was significantly elevated above HC levels ($p$s $< .01$) with the exception of EtOH-SAL for *Arc*; and EtOH-SAL and EtOH-PHY for *egr-1* expression ($p > .05$).

In the dHPC (**Fig. 8.3B**), no main of interaction effects involving alcohol (dosing) were found for any IEG and a main effect of drug was found only for *c-Fos* (**Table 8.2**). The EtOH-PHY and SI-PHY groups showed significantly higher *c-Fos* expression than their saline-treated counterparts ($p < .01$), with no difference between the two groups ($p > .40$). Dunnett's tests revealed that SI and EtOH rats given either drug had significantly elevated expression of all IEGs above HC levels. Finally, in the vHPC (**Fig. 8.3C**), there were no main or interaction effects of dosing or drug (**Table**

**8.2**). While Dunnett's tests revealed that all four treated groups had significantly higher expression of *c-Fos* and *Arc* than HC levels (*p*s < .05), there was no elevation above HC in vHPC *egr-1* and *Npas4* (*p*s > .10).

Taken together, the results of Experiment 8.2 demonstrate that systemic PHY administration prior to context preexposure in the CPFE rescues context freezing in ethanol-exposed rats. A similar effect on IEG expression was found only for *c-Fos* in mPFC. PHY administration increased prefrontal *Arc* and *Npas4* expression, and dHPC *c-Fos* expression regardless of alcohol treatment. Consistent with previous findings (Heroux et al., 2019) alcohol impaired IEG expression only in mPFC.

| | Medial Prefrontal Cortex (mPFC) | | | | Dorsal Hippocampus (dHPC) | | | | Ventral Hippocampus (vHPC) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *F* | *p* | *Final n* | *Outliers* | *F* | *p* | *Final n* | *Outliers* | *F* | *p* | *Final n* | *Outliers* |
| **Genes** | | *(HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY)* | | | | *(HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY)* | | | | *(HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY)* | | |
| ***c-Fos*** | | | | | | | | | | | | |
| *Dosing* | 4.53 | < .05 | | | 0.58 | > .45 | | | 0.73 | > .39 | | |
| *Drug* | 7.63 | < .01 | 23, 11, 8, 12, 12 | 1, 1, 1, 0, 0 | 21.59 | < .01 | 21, 10, 10, 11, 11 | 2, 1, 1, 0, 0 | 1.53 | > .23 | 22, 10, 10, 11, 9 | 2, 1, 1, 1, 0 |
| *Dosing × Drug* | 7.56 | < .01 | | | 0.12 | > .70 | | | 4.37 | < .05 | | |
| ***Arc*** | | | | | | | | | | | | |
| *Dosing* | 20.26 | < .01 | | | 0.11 | > .74 | | | 0.01 | > .90 | | |
| *Drug* | 18.13 | < .01 | 22, 11, 9, 10, 11 | 2, 1, 0, 2, 1 | 0.1 | > .74 | 21, 10, 10, 10, 10 | 2, 1, 1, 1, 1 | 1.55 | > .22 | 22, 10, 10, 11, 10 | 2, 1, 1, 1, 1 |
| *Dosing × Drug* | 0.04 | > .80 | | | 0.03 | > .87 | | | 1.71 | > .19 | | |
| ***Egr-1*** | | | | | | | | | | | | |
| *Dosing* | 12.03 | < .01 | | | 0.54 | > .45 | | | 0.14 | > .70 | | |
| *Drug* | 2.94 | > .10 | 22, 11, 9, 11, 10 | 2, 1, 0, 1, 2 | 2.83 | > .10 | 20, 11, 10, 10, 10 | 1, 1, 1, 1, 1 | 1.85 | > .18 | 22, 10, 10, 11, 10 | 2, 1, 1, 1, 1 |
| *Dosing × Drug* | 2.51 | > .12 | | | 2.75 | > .10 | | | 0.08 | > .77 | | |
| ***Npas4*** | | | | | | | | | | | | |
| *Dosing* | 19.2 | < .01 | | | 0.22 | > .64 | | | 0.35 | > .56 | | |
| *Drug* | 9.55 | < .01 | 23, 12, 9, 11, 11 | 1, 0, 0, 1, 1 | 0.49 | > .48 | 21, 11, 11, 11, 11 | 2, 0, 0, 1, 0 | 0.07 | > .79 | 22, 10, 10, 12, 10 | 2, 1, 1, 0, 1 |
| *Dosing × Drug* | 0.49 | > .48 | | | 0.01 | > .90 | | | 0.43 | > .50 | | |

Table 8.2 Final group numbers (*n*), number of outliers removed (*HC, SI-SAL, SI-PHY, EtOH-SAL, EtOH-PHY*), and statistical results for all factorial ANOVAs (see *F* and *p* values) for each gene (*c-Fos*, *Arc*, *Egr-1*, and *Npas4*) in each region (mPFC, dHPC, and vHPC) for Experiment 8.2.

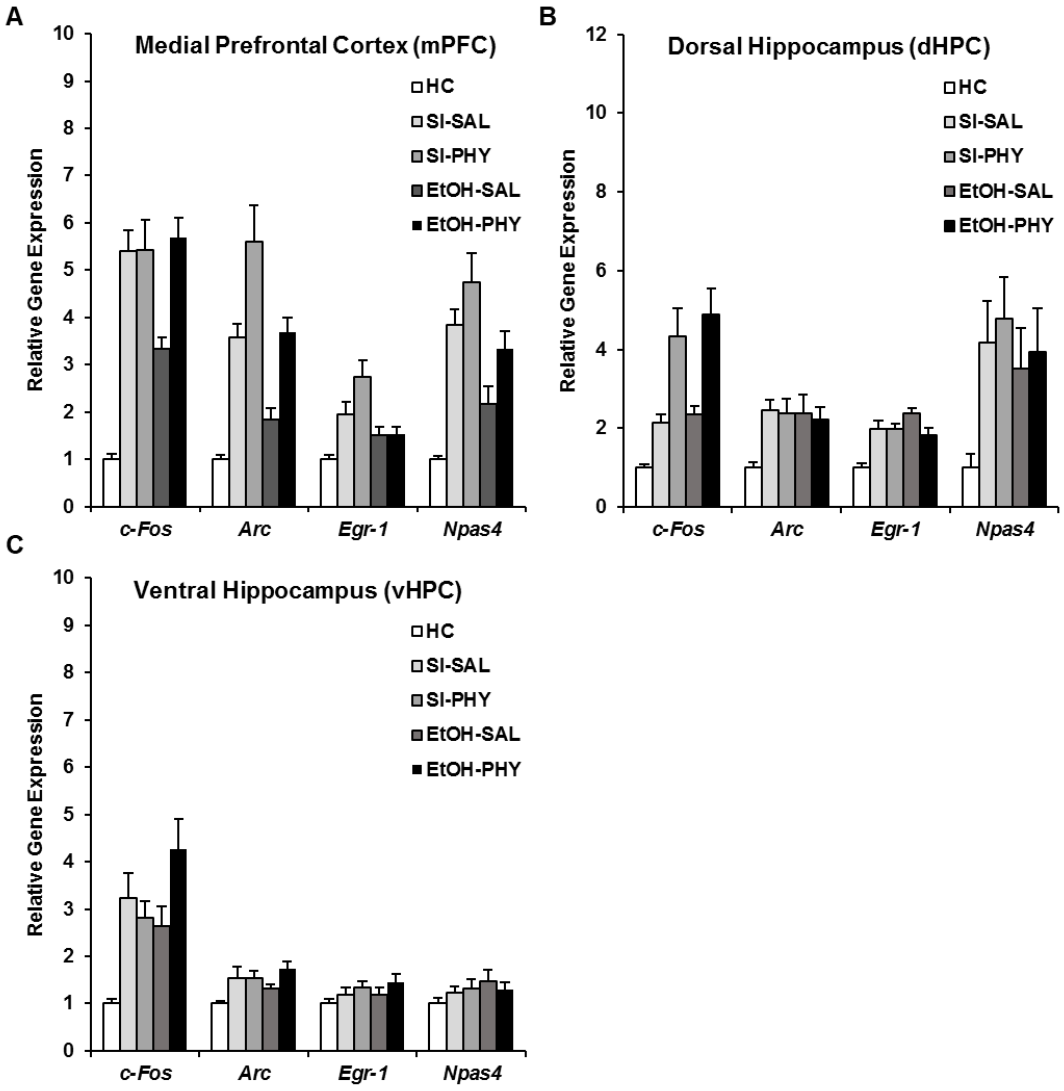


Figure 8.3 mRNA expression of *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC **(A)**, dHPC **(B)**, or vHPC **(C)** in SI and EtOH rats treated with SAL or PHY sacrificed 30 min after context exposure in the CPFE. **(A)** The EtOH-SAL group showed significantly reduced mRNA expression of every IEG compared to Group SI-SAL. PHY treatment rescued *c-Fos* expression in the mPFC of EtOH rats. **(B)** PHY treatment elevated *c-Fos* expression in the dHPC in both SI and EtOH rats. **(C)** PHY treatment elevated *c-Fos* expression in the vHPC in EtOH rats.

## 8.4 Discussion

The current set of experiments examined the effects of systemic PHY on disruptions in contextual fear conditioning and regional IEG expression in adolescent rats receiving neonatal ethanol exposure during the brain growth spurt. Consistent with our prior reports (Heroux, Robinson-Drummer, et al., 2019; Murawski et al., 2012; Murawski & Stanton, 2010, 2011), high binge-like doses of ethanol given over PD4-9 abolished both post-shock and retention test freezing in the CPFE. This behavioral disruption in ethanol-exposed rats was rescued by PHY administration prior to all three phases (Experiment 8.1) or just prior to context preexposure (Experiment 8.2), with no effects of PHY on behavior of sham-intubated rats. Furthermore, PHY treatment prior to context learning selectively rescued ethanol-induced disruptions in prefrontal expression of *c-Fos* but not *Npas4* or *Arc,* or *Egr-1*. Prefrontal expression of *Npas4* and *Arc* was non-specifically boosted by PHY in both ethanol-exposed and sham-intubated rats, whereas PHY increased *Egr-1* in sham but not ethanol-exposed rats. Hippocampal IEG expression was not impaired by alcohol or altered by PHY, except for increased *c-Fos* expression in dHPC regardless of alcohol exposure. Taken together, augmenting cholinergic signaling rescues neonatal-alcohol induced impairment of configural learning, memory, and prefrontal gene expression.

The present study informs the psychological and neural mechanisms through which neonatal alcohol impairs cognition. As reviewed in **Chapter 1**, the CPFE develops between PD17 and PD24 in the rat, after which it depends on activity and cholinergic muscarinic-receptor cell signaling in the dHPC and mPFC during all three phases (Heroux et al., 2017; Jablonski et al., 2012; Robinson-Drummer et al., 2016, 2017; Schiffino et al., 2011). While impairment of the CPFE is robust across dosing

scenarios, neonatal ethanol exposure has no effect on single-trial sCFCwhich is known to be hippocampal dependent (Wiltgen et al., 2006; Zelikowsky et al., 2013). Neonatal ethanol exposure impairs the acquisition and/or consolidation of the conjunctive context representation on the preexposure day of the CPFE (Goodfellow & Lindquist, 2014; Heroux, Robinson-Drummer, et al., 2019), resulting in abolished post-shock and retention test freezing [see Experiment 8.1 and 8.2]. Moreover, ethanol-exposed rats have significantly reduced prefrontal but not hippocampal immediate early gene expression during context learning in the CPFE [Heroux et al., 2019; see **Chapter 7**]. In summary, neonatal alcohol impairs incidental context learning or consolidation through a mechanism that may involve reduced prefrontal activity or plasticity.

In the current study, systemic administration of PHY prior to all three phases or just prior to context preexposure rescued post-shock and retention test freezing deficits in ethanol-exposed rats. This extends our prior finding that PHY treatment prior to all three phases rescues retention freezing in PD7-9 ethanol-exposed rats (Dokovna et al., 2013). In both cases, the behavioral rescue was specific to ethanol-exposed rats, without any non-specific boost in performance in sham-intubated rats. In addition to rescuing behavioral performance, PHY treatment rescued prefrontal *c-Fos* expression in ethanol-exposed rats while boosting prefrontal *Arc* and *Npas4* and hippocampal *c-Fos* expression in both dosing groups. These behavioral findings support previous work showing that deficits in trace fear conditioning in rats receiving PD4-9 ethanol exposure are also dose-dependently rescued by systemic PHY treatment prior to training (Hunt & Barnet, 2015). While more research is needed, some mechanisms by which PHY treatment may rescue neurobehavioral deficits include decreasing neuroinflammation, lowering the threshold for LTP induction, and

increasing bioavailability of acetylcholine (Goodfellow et al., 2018; Kalb et al., 2013; Monk et al., 2012; Pyapali, Turner, Williams, Meck, & Swartzwelder, 1998). Despite our results suggesting selective prefrontal targeting by alcohol, they do not discount previous research showing robust cholinergic dysfunction in the hippocampus resulting from alcohol exposure, they only question whether this dysfunction is exclusively responsible for cognitive deficits (see **Section 3.3** and **3.5** in **Chapter 3** for full discussion). Nevertheless, taken together, the results in this chapter further support our conclusion that impaired context learning or consolidation by PD4-9 ethanol exposure reflects impaired prefrontal activity or plasticity involving cholinergic signaling.

The current study supports and extends previous literature examining the neurobiology of contextual fear conditioning and the role of IEGs in learning and memory. As reviewed in **Chapter** 2, we chose to examine the IEGs *c-Fos*, *Arc*, *Egr-1*, and *Npas4* because of their involvement in synaptic plasticity supporting LTM formation and consolidation; and because neonatal ethanol exposure impairs expression of these IEGs during context learning (**Chapter 7**; Jablonski et al., 2018). Despite emerging evidence supporting a role of the prefrontal circuitry across learning paradigms, especially those relevant to alcohol-induced insult (Gilmartin et al., 2014; Giustino & Maren, 2015; Heroux, Robinson-Drummer, et al., 2019; Heroux et al., 2017; Ramanathan et al., 2018; Zelikowsky et al., 2014), examination of the role of prefrontal IEG-expressing neurons in memory has been largely ignored in favor of the hippocampus. Although the mechanism is not known, we show that PHY treatment in ethanol-exposed rats elevates expression of *c-Fos*, *Arc*, and *Npas4* in the prefrontal cortex bot only *c-Fos* in the hippocampus. PHY treatment reversed the alcohol-

induced deficit in context fear and in prefrontal *c-Fos* expression. For other prefrontal IEG expression as well as for dHPC *c-Fos* expression, PHY treatment elevated IEG expression but did not rescue the alcohol-induced deficit. While the current study cannot fully establish causality between gene expression and behavior, the data suggest a mechanistic role for prefrontal *c-Fos* expression. Increased acetylcholine bioavailability via systemic PHY treatment elevates *c-Fos* expression by activating neuronal nicotinic and muscarinic receptors across receptor-populated brain regions, including the prefrontal cortex (Kaufer, Friedman, Seidman, & Soreq, 2016; Pongrac & Rylett, 2003; Thomsen et al., 2008). While there is a clear disruption in dHPC cholinergic receptor function after neonatal alcohol exposure in rats, this disruption likely extends beyond the dHPC and might inform our findings in the mPFC (Monk et al., 2012). Given that *c-Fos* expression is linked to neuronal activity and plasticity necessary for cellular consolidation of long-term memory (Gallo et al., 2018; Minatohara et al., 2016), PHY treatment likely recues alcohol-induced deficits by augmenting plasticity-related protein expression. It is also possible that elevated prefrontal *Arc* and hippocampal *c-Fos* expression in ETOH-PHY rats contributed to the rescue by exceeding a threshold of expression that was not met in EtOH-SAL rats. Another contributing factor might be IEG expression differences between neuronal sub-regions (e.g., IL vs. PL) and sub-types between dosing conditions, which may contribute to the cholinergic rescue of neurobehavioral effects of alcohol. More research is needed to establish mechanistic roles of cholinergic signaling, regional expression of specific IEGs, and behavioral performance in normally developing and ethanol-exposed rats.

In summary, our findings demonstrate that the acetylcholinesterase inhibitor physostigmine rescues ethanol-induced disruptions in context memory and prefrontal expression of some immediate early genes in adolescent rats. While the current study cannot establish a causal link between IEG expression and behavior, these results suggest that ethanol disrupts behavioral performance by altering activity and/or plasticity induced by cholinergic signaling in the prefrontal cortex during configural learning and memory. Taken together with our recent reports (Dokovna et al., 2013; Heroux, Robinson-Drummer, et al., 2019; Jablonski et al., 2018), these findings are important because prefrontal dysfunction is an integral hallmark of FASD in humans, but animal models have thus far largely failed to capture prefrontal dysfunction after third-trimester equivalent exposure. Building upon an emerging body of animal and human research linking alcohol and cholinergic dysfunction, future experiments should characterize the effects of intra-cranial infusions of drugs that augment cholinergic signaling into mPFC or other discrete brain regions on behavioral performance of alcohol-exposed rats across the lifespan.

# Chapter 9

# SUMMARY AND CONCLUSIONS

This dissertation examined the neural and behavioral mechanisms of context and contextual fear learning and memory in the CPFE and how these mechanisms are disrupted by third-trimester equivalent alcohol exposure in rats. Experiments 6.1-6.2 examined prefrontal and hippocampal mechanisms of incidental context learning and memory during context preexposure in the CPFE. Experiments 7.1-7.2 examined the effects of PD4-9 alcohol exposure on post-shock and retention test freezing in the CPFE and sCFC. Experiment 7.3 examined the effects of this exposure on regional molecular activity during context preexposure in the CPFE. Finally, Experiments 8.1-8.2 examined the efficacy of the acetylcholinesterase inhibitor physostigmine in rescuing impaired regional molecular activity and behavioral performance in alcohol-exposed rats in the CPFE. In the following discussion I summarize the rationale, hypotheses, findings, and future directions for these experiments and briefly discuss them in relation to the neurobiology of CFC and rat models of FASD.

## 9.1 Prefrontal and Hippocampal Mechanisms of Context and Contextual Fear Learning and Memory in the CPFE

The rationale for the experiments in this dissertation largely stems from our recent discovery of a necessary role of the prefrontal cortex across all three phases of the CPFE variant of Pavlovian CFC (see **Section 4.2** in **Chapter 4**; Heroux et al., 2017). These findings lie in stark contrast to the general lack of an effect of prefrontal inactivation or lesions on performance of sCFC besides increased fear generalization

(Giustino & Maren, 2015; Xu & Südhof, 2013). While a modulatory role of prefrontal plasticity in the consolidation of context-shock associations has been previously established (Rozeske et al., 2015), our study provided novel evidence of prefrontal circuitry underlying the acquisition and/or consolidation of a context representation in the absence of aversive stimuli (Heroux et al., 2017; Robinson-Drummer et al., 2017). Due to a similar dependence of the CPFE on hippocampal activity, we hypothesized that the mPFC interacts with the HPC via reciprocal connectivity with the VMT to support the encoding or consolidation of a long-term conjunctive context representation in the CPFE (see **Section 2.1.3** in **Chapter 2**; Heroux et al., 2017). Accordingly, in **Chapter 6**, we first asked the question: *does prefrontal inactivation effect molecular activity in the ventral midline thalamus and hippocampus during incidental context exposure?* In Experiment 6.1, we found that prefrontal inactivation disrupted IEG expression in the mPFC, VMT, and VHPC but not dHPC during context preexposure. We then asked the question: *Does ventral hippocampal inactivation effect molecular activity in the prefrontal cortex and dorsal hippocampus during incidental context exposure?* In Experiment 6.2, we found that ventral hippocampal inactivation disrupted IEG expression in the vHPC, mPFC, and dHPC during this preexposure. These results did not support our initial mPFC-dHPC interaction hypothesis, as prefrontal inactivation did not alter dHPC activity during context exposure. Instead, to our knowledge, this is the first study to suggest a role of circuitry between the mPFC and vHPC in incidental context learning and memory during the CPFE.

It's informative to compare these results to recent studies and models of prefrontal circuitry underlying CFC (see **Section 2.1.2** in **Chapter 2**; Gilmartin,

Balderston, & Helmstetter, 2014; Giustino & Maren, 2015; Rozeske, Valerio, Chaudun, & Herry, 2015). In particular, Rozeske et al.'s (2015) model suggests a modulatory role of the PFC in the context specificity and consolidation of the context-shock association. In this model, during context encoding, the HPC sends contextual information to the PFC, which regulates feature encoding and specificity of the context representation via projections to the NR and then HPC (Ramanathan et al., 2018; Rozeske et al., 2015; Xu & Südhof, 2013). This contextual information is sent to the amygdala for the acquisition of the context-shock association and is then sent to the AC, which in turn modulates consolidation and fear expression. In contrast, unlike in sCFC (see **Section 2.1.1** in **Chapter 2**), we report no compensation occurs after mPFC or vHPC inactivation in the CPFE (Heroux et al., 2017; Matus-Amat et al., 2007). It's unlikely that the PFC is *required* for encoding contextual cues in the CPFE as rats can acquire a context-shock association in sCFC under PFC inactivation (Giustino & Maren, 2015; Heroux et al., 2017). Rozeske et al.'s (2015) model would predict that prefrontal inactivation prior to context preexposure would not impair the CPFE *per se*, but instead result in a less precise context representation and thus more generalized fear when tested in a non-conditioned context. While our data doesn't directly support this, it's possible to reconcile this difference when considering that, unlike sCFC, the CPFE requires a configural context representation to be associated with foot-shock (Jablonski et al., 2012; Rudy & O'Reilly, 1999b). Therefore, via downstream effects on NR and vHPC, this prefrontal inactivation may result in a less feature-rich conjunctive context representation, subsequently interfering with rapid pattern completion and thus context retrieval during immediate-shock training on the next day. One way to test this hypothesis could be by taking advantage of the finding

that, in the CPFE, the context representation does not undergo reconsolidation after retrieval unless a biologically significant event occurs such as introducing novel stimuli (Biedenkapp & Rudy, 2004). Thus, if rats under prefrontal inactivation acquire a less feature-rich representation, then reexposure to the context should trigger reconsolidation to update with features that were originally missed, rendering the memory susceptible to protein synthesis inhibition. Therefore, interfering with PFC or HPC during a second context preexposure should impair the CPFE only in rats that were under prefrontal inactivation during the first preexposure. This general interpretation would be consistent with studies done by Xu & Südhof (2013) and Ramanathan et al. (2018), which suggest that PFC-NR connectivity modulates the precision of hippocampal-dependent context representations during CFC (see **Section 2.1.2** in **Chapter 2**). One important caveat is that it's unclear if the roles of the mPFC and NR would change when contextual learning is incidental vs. driven by shock reinforcement, as these previous studies fail to dissociate context from contextual fear learning. If the role of the NR is in specificity of context encoding but not contextual fear *per se*, then inactivation of NR (or PFC) during training should have no effect on the CPFE as the context was encoded on the previous day. While we've shown that pre-training inactivation of the PFC does not impair fear acquisition, it's unknown if this manipulation caused generalization to the alternate context (Heroux et al., 2017). These are all fruitful directions for future research, especially because the CPFE dissociates these component processes of CFC.

These recent studies from our lab (see **Chapter 4)** and others motivate the first working model of the neurobiology of the CPFE variant of CFC (see **Figure 9.1** and **9.2**). Incorporating prefrontal circuitry, this model significantly expands upon earlier

work that almost exclusively focused on hippocampal mechanisms (Matus-Amat et al., 2004; Rudy, 2009; Rudy et al., 2002; Rudy & O'Reilly, 1999a). The proposed neurobiology underlying discrete processes that occur during context preexposure can be seen in **Figure 9.1**. We propose that encoding of contextual features occurs within the PHR before being relayed directly to the dorsal and indirectly to the ventral HPC. This contextual information is sent to the mPFC via direct projections from the vHPC, which in turn projects back to the vHPC via NR. These specific pathways would be supported by the results presented in Chapter 6, in which vHPC inactivation disrupted mPFC but not VMT IEG expression, but mPFC inactivation disrupted VMT and vHPC IEG expression. The former suggests a direct projection, and the latter suggests indirect effects of vHPC activity via the NR, in the absence of any direct mPFC-HPC projections in rats. It's also possible that that vHPC inactivation alters mPFC indirectly by impairing dHPC activity and thus any projections from the dHPC to the AC that convey contextual information, although it's unlikely that the dHPC would need vHPC input to achieve this (Fanselow & Dong, 2010). Moreover, these prefrontal projections to the NR likely contribute to the specificity of the context representation via modulating the number of features that get incorporated into the conjunctive context representation (Ramanathan et al., 2018; Xu & Südhof, 2013). Amygdalar projections to the mPFC originating from the vHPC might communicate cue saliency during exposure (Gilmartin et al., 2014; Sengupta et al., 2018), and thus may modulate this incorporation process. Context preexposure requires BLA activity but not plasticity, suggesting a role of this region during encoding (Huff & Rudy, 2004). Indeed, Kim & Cho (2017) showed that a subset of vHPC neurons projecting to the amygdala and mPFC preferentially respond to context exposure, and have

excitatory influences on these structures during context encoding. In any case, after receiving information about features from the NR, the vHPC sends this contextual information back to the dHPC indirectly through the PHR. Finally, consolidation of the conjunctive context representation is supported by dorsal and ventral hippocampal communication, as pharmacological disruption of these regions pre- or post- context preexposure impairs the CPFE (Chang & Liang, 2012; Cullen et al., 2017; Matus-Amat et al., 2007; Robinson-Drummer et al., 2016; Rudy & Matus-Amat, 2005; Schiffino et al., 2011).

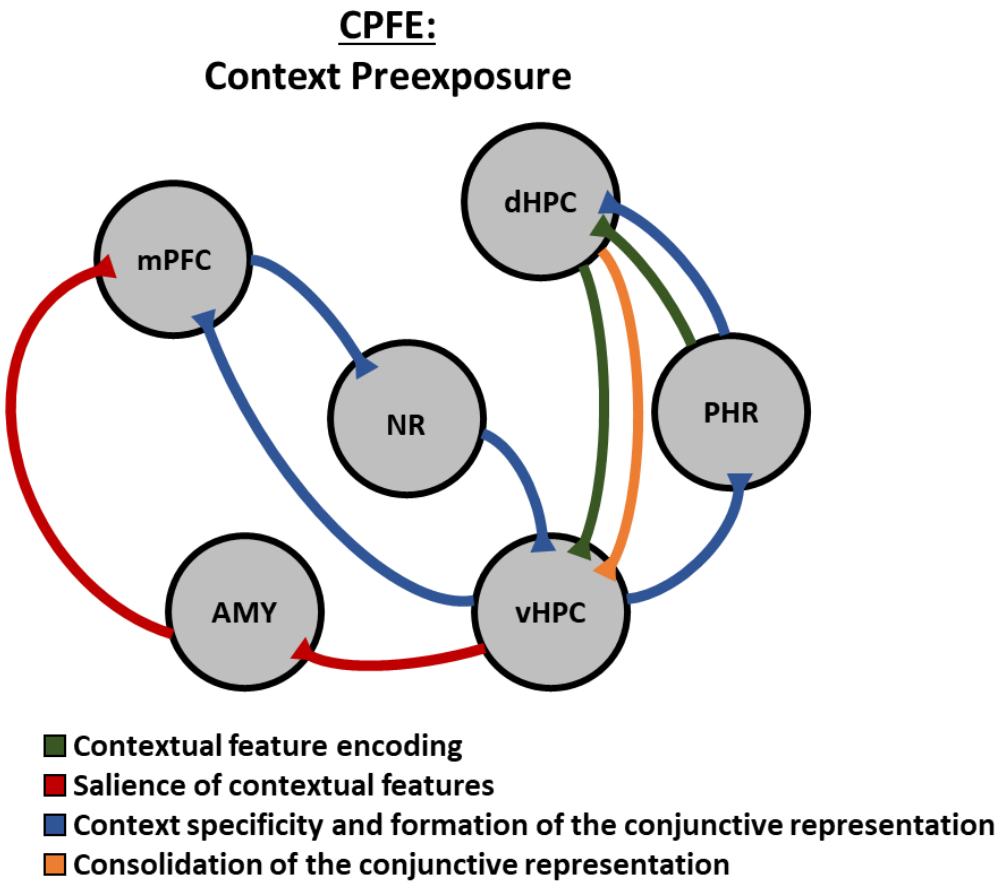


Figure 9.1 **Hypothetical model of the neurobiology underlying discrete processes occurring during Context Preexposure in the CPFE.** Upon chamber entry, information about contextual features is sent to the dHPC and vHPC from the PHR. The vHPC sends this contextual information to the mPFC, which in turn projections back to the vHPC via the NR. This mPFC-NR pathway is important for the specificity of the context representation, and dictates which features get incorporated into the context representation. This process is modulated by BLA input into the mPFC, which likely has a role in instructing the mPFC about the saliency of contextual features. Consolidation of the conjunctive context representation occurs within hippocampal circuitry after the vHPC relays which features are to be bound into the representation.

The proposed neurobiology underlying immediate-shock training can be seen in **Figure 9.2**. We propose that retrieval of the context representation occurs via PHR responding to a feature upon chamber entry and causing pattern completion within the HPC, likely via CA3 recurrent collateral activation (Rolls, 2013; Rudy, 2009). The vHPC then projects this conjunctive context representation to the BLA where it is associated with immediate foot-shock (Matus-Amat et al., 2007; Miller et al., 2019). This association is expressed via CeA projections to the ventrolateral PAG. The mPFC is not required for any of these processes, as prefrontal inactivation during training impairs retention test freezing but leaves context retrieval, context-shock encoding, and post-shock freezing intact (Heroux et al., 2017). The mPFC likely contributes to the consolidation of the context-shock association. This role of the PFC is consistent with Rozeske et al.'s (2015) model but may also reflect this region's involvement in memory reconsolidation or, in this case, updating a neutral context representation to include foot-shock (Stern et al., 2014). Nevertheless, this prefrontal modulation of consolidation likely occurs via projections to the BLA, which in turn projects back to the vHPC. In support of this, Huff et al. (2016) demonstrated that silencing BLA projections to the vHPC after immediate-shock training but not context preexposure impairs retention test freezing in the CPFE. Taken together, this model emphasizes recent discoveries of involvement of mPFC and vHPC circuitry in the CPFE. Importantly, this model is general and largely hypothetical, with its purpose being to encourage future hypothesis testing using temporally-precise and pathway-specific circuit manipulation during discrete behavioral processes (see **Section 9.3** below).

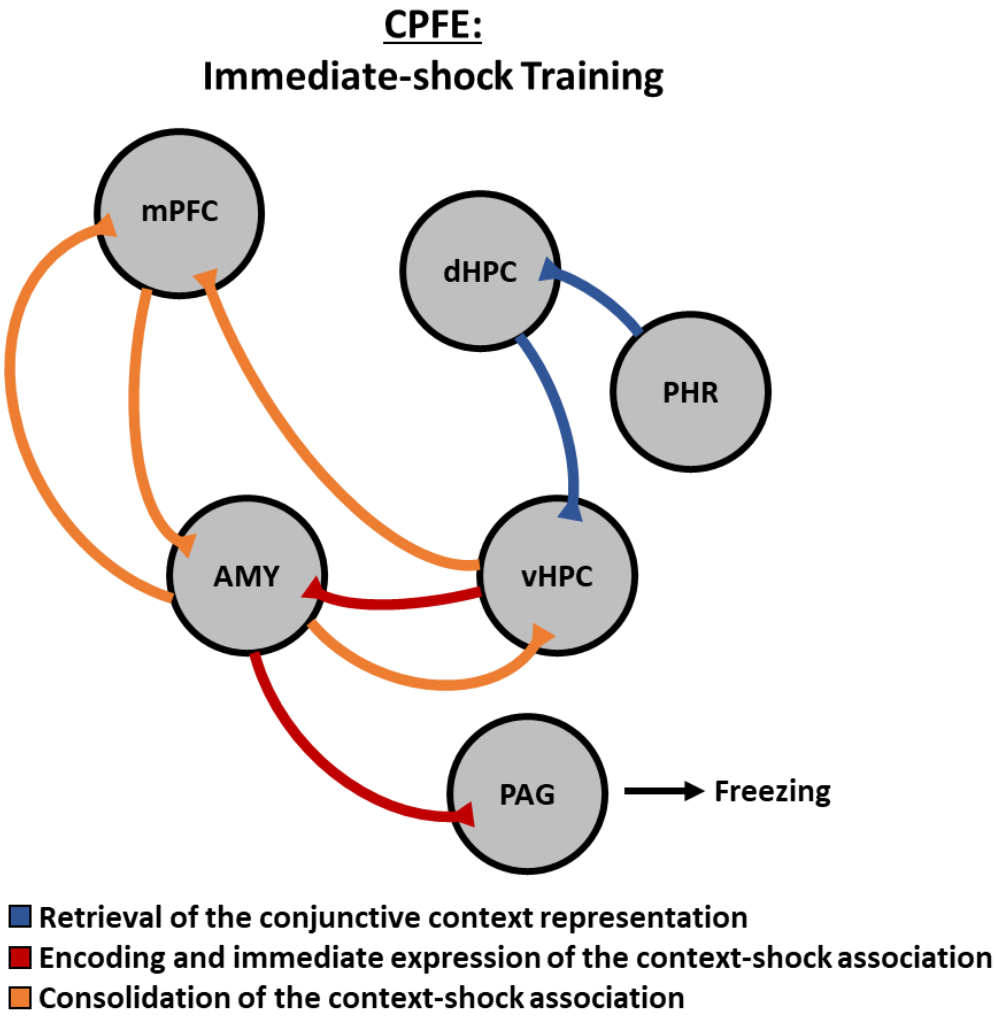


Figure 9.2 **Hypothetical model of the neurobiology underlying discrete processes occurring during Immediate-shock Training in the CPFE.** Upon chamber entry, PHR responds to features of the context and drives hippocampal pattern completion and thus context retrieval via projections to the dHPC and activation of recurrent collaterals in the CA3 sub-region. The vHPC sends this context representation to the BLA, where it converges with foot-shock US inputs to support the formation of the context-shock association. Immediate expression of the context-shock association via the species-typical freezing response occurs via direct projections between the CeA and the ventrolateral PAG. Consolidation of this association is supported by ventral hippocampal and amygdalar connectivity with the mPFC.

## 9.2 Acute Enhancement of Cholinergic Function Rescues Neurobehavioral Disruption after Neonatal Alcohol Exposure in Rats

As reviewed in Chapter 3, PD4-9 alcohol exposure abolishes retention test freezing in the CPFE but leaves cued and un-signaled fear conditioning intact in rats (Murawski et al., 2012; Murawski & Stanton, 2010). Our lab has shown that this exposure results in CA1 pyramidal cell loss and a knockdown of hippocampal *c-Fos* protein expression during context preexposure, which could underlie disrupted context learning and/or memory (Murawski et al., 2012). Despite this, these previous studies are unable to dissociate PD4-9 alcohol effects on preexposure or training day processes because only retention-test freezing was measured. Post-shock freezing helps inform which of these processes is impaired (Chapter 1, Jablonski et al., 2013) and PD7-9 alcohol appears to selectively impair retention-test freezing (Jablonski & Stanton, 2014). Accordingly, in Chapter 7, we first asked the question: *does PD4-9 alcohol exposure disrupt incidental context learning and memory during context preexposure or contextual fear learning and memory during immediate-shock training in the CPFE?* In Experiment 7.1, alcohol exposure impaired both post-shock and retention test freezing in the CPFE. These results are still unable to determine if alcohol-exposed rats are unable to acquire, consolidate, or retrieve a context representation or if these processes are intact but they are unable to acquire a context-shock association during training. To examine these possibilities in another paradigm, we asked the question: *does PD4-9 alcohol exposure impair context or contextual fear learning in sCFC?* In Experiment 7.2, alcohol-exposed rats had intact retention test freezing in single-trial sCFC. This result indicated that: (1) alcohol-exposed rats can acquire a context representation and associate it with shock, (2) potential "performance effects" of alcohol exposure such as reduced feature perception or shock

sensitivity do not underlie deficits in the CPFE, and (3) these rats are unimpaired in a hippocampal-dependent (Wiltgen et al., 2006) but prefrontal-independent (Heroux et al., 2017) variant of CFC. Therefore, we hypothesized that PD4-9 exposure results in prefrontal dysfunction and impaired consolidation of the conjunctive context representation in the CPFE. Finally, the third question we asked was: *does neonatal alcohol exposure alter molecular activity in the prefrontal cortex and hippocampus during context preexposure in the CPFE?* In Experiment 7.3, alcohol exposure impaired expression of the IEGs *c-Fos*, *Arc*, *Egr-1*, and *Npas4* in the mPFC but not dHPC during context exposure. These data support our hypothesis that prefrontal impairments underlie alcohol-induced CPFE deficits that were historically attributed to hippocampal impairment(s). These results demonstrate that third-trimester-equivalent alcohol exposure disrupts behaviorally-driven molecular activity in the prefrontal cortex that may subserve incidental contextual memory processes in rats.

As reviewed in Chapter 3, neonatal alcohol exposure causes both acute and persistent disruptions in cholinergic signaling important for successful neurodevelopment and cognition across the lifespan in rats. Developmental choline supplementation mitigates alcohol-induced disruptions in MWM, CFC, trace fear conditioning, and spatial working memory (Hunt & Barnet, 2015; Idrus et al., 2017; Ryan et al., 2008; Thomas et al., 2009, 2007, 2004, 2010; Wagner & Hunt, 2006). This treatment is proving to be at least partially efficacious in rescuing cognitive function in developing children with FASD (Jacobson et al., 2018; Wozniak et al., 2015, 2013). One major question that emerges from this literature is whether acutely enhancing cholinergic signaling later after exposure would also rescue impaired cognition. Our lab has previously shown that systemic administration of the

acetylcholinesterase inhibitor physostigmine prior to every phase rescues deficits in retention test freezing in the CPFE in PD7-9 alcohol-exposed rats (Dokovna et al., 2013). The exact phase of the CPFE that mediates this rescue effect and whether the PD7-9 result would generalize to the more severe PD4-9 exposure window is unclear. Therefore, in Chapter 8, we asked the question: *does acute physostigmine treatment prior to every phase or just during context preexposure rescues PD4-9 alcohol-induced deficits in the CPFE?* In Experiment 8.1, alcohol-exposed rats given physostigmine prior to every phase showed normalized post-shock and retention test freezing relative to sham-intubated rats, which did not benefit from treatment. Given the dependence of preexposure day processes on cholinergic signaling in the mPFC and dHPC (see Chapter 4; Robinson-Drummer et al., 2016, 2017), we then hypothesized that this treatment prior to just context exposure would rescue impaired performance and IEG expression in alcohol-exposed rats. Therefore, we asked: *Can alcohol-induced neurobehavioral disruptions be rescued by enhancing cholinergic signaling via acute acetylcholinesterase inhibitor treatment prior to context exposure?* In Experiment 8.2, physostigmine treatment prior to just context preexposure rescued the CPFE in alcohol-exposed rats. This treatment specifically rescued prefrontal *c-Fos* expression, in tandem with non-specific elevations in mPFC *Arc* and *Npas4* as well as dHPC and VHPC *c-Fos* in both dosing groups. Future studies are needed to establish regional specificity of this rescue (i.e., whether intra-mPFC physostigmine prior to context preexposure produces the same effect). Such studies would address the critical issue of whether there is a causal link between regional alterations in activity or cholinergic signaling and impaired cognition across in this rodent model of FASD.

The results of these two research aims have several broad implications for developmental FASD research in humans and animal models. The results of **Chapter 7** highlight the need, as a field, to re-examine alcohol-induced deficits that have been attributed solely to hippocampal dysfunction in behavioral tasks that require both the dHPC and mPFC (see **Chapters 3 and 7** for extended discussion). Indeed, in part because the CPFE was largely used as a "hippocampal-dependent" CFC task, we previously attributed deficits in the CPFE almost solely to reduced hippocampal molecular activity and cell counts (G. F. Hamilton et al., 2011; Murawski et al., 2012; Murawski & Stanton, 2010). While our current results do not rule out hippocampal involvement, they do suggest a major role of impaired prefrontal molecular and cholinergic activity in cognitive deficits arising from third-trimester equivalent alcohol exposure in rats. These findings are particularly important given evidence showing alcohol deficits are the largest in tasks that engage both the HPC and PFC, such as in trace fear conditioning (DuPont et al., 2014; Gilmartin et al., 2014; Goodfellow et al., 2016; Hunt et al., 2009; Wagner & Hunt, 2006). This pattern is especially interesting given that it was recently discovered that this alcohol exposure results in a dramatic (>20%) reduction in neurons in the NR, which serves as the major communication nexus between these two regions (Gursky, Savage, & Klintsova, 2019). Taken together with our proposed model (see **Figure 9.1**), it's likely that PD4-9 alcohol exposure impairs the CPFE by disrupting communication between the mPFC and vHPC. The results of **Chapter 8** demonstrate that acute treatment with drugs that enhance cholinergic system function later in development is sufficient to rescue select neurobehavioral deficits in rat models of FASD. This finding is important for two reasons. First, this finding complements only two other studies that have examined

acute treatment with acetylcholinesterase inhibitors in exposed rats (Dokovna et al., 2013; Hunt & Barnet, 2015). Importantly, this is the first study to suggest a neural mechanism of this rescue in enhanced prefrontal activity via increased activation of muscarinic acetylcholine receptors, which are required for the CPFE (Heroux, Horgan, Rosen, et al., 2019; Robinson-Drummer et al., 2016, 2017). Second, these results suggest that acute enhancement of cholinergic function might be efficacious in attenuating select cognitive deficits in humans with FASD. This demands further testing, as currently the alcohol field is focused on using cholinergic treatments (e.g., choline supplementation) concurrent with or soon after alcohol exposure but not later in life (Jacobson et al., 2018; Wozniak et al., 2015, 2013). Collectively, this research promises to shed light on the basic neural mechanisms underlying cognitive impairment resulting from developmental alcohol exposure.

## 9.3 Future Directions

### 9.3.1 Limitations

The neurobiological analyses in this dissertation are subject to several important limitations discussed in the previous experimental chapters. Notably, these limitations include 1) examination of mRNA instead of protein expression, 2) dissecting out entire brain regions vs. individual neural sub-regions, 3) inability to determine cell-type specific effects, and 4) assessing only one time point (30min) after experimental manipulations. Furthermore, we are unable to determine the specific function of the observed IEG expression in supporting behavior in the absence of loss- and gain-of-function experiments that directly manipulate specific gene expression during the CPFE. While we measured the expression of several IEGs (*c-Fos*, *Arc*, *Egr-*

*1*, and *Npas4*), patterns of expression of these genes generally did not differ across the qPCR experiments. A notable exception to this is the lack of *Arc, Egr-1,* and *Npas4* expression seen in the VMT when compared to robust *c-Fos* expression observed in this area. This may suggest that plasticity in this region is not heavily engaged by context exposure, as *c-Fos* expression has been tied more heavily to neural activity but not plasticity supporting LTM (see **Section 2.2** in **Chapter 2** for more discussion). Another exception can be seen in the effects of physostigmine on IEG expression in SI and EtOH rats, which was more heavily tied to a boost in *c-Fos* and *Arc* expression (see **Section 8.4** in **Chapter 8** for specific discussion). Despite these limitations, the methodology in this dissertation permits measurement of several IEGs. This provides novel normative and pharmacological data concerning cholinergic and GABAergic modulation of IEG expression during behavioral experience (context exposure). Pursuing these findings with other methodologies that address the limitations mentioned above is an obvious and important direction for future research. Finally, another important future direction is to determine whether similar neurobehavioral impairments are seen after exposure to other teratogens (e.g., such as nicotine and marijuana) during the same developmental window in rats.

### 9.3.2 Experimental Predictions

Several experimental predictions can be made when considering the findings of all three aims of this dissertation and our newly proposed model of the neurobiology of the CPFE (see **Figure 9.1** and **9.2**). Some of these predictions, especially those concerning the specific roles of the mPFC and NR during incidental context encoding and consolidation appear in **Sections 2.1.2 and 2.1.3** in **Chapter 2** and **Section 9.1** in **Chapter 9** . Our model predicts that pre-preexposure but not pre-

training inactivation of NR would impair the CPFE by causing a feature-impoverished context representation (Xu & Südhof, 2013). While inactivation of mPFC prior to either phase disrupts 24-hr retention of the CPFE (Heroux et al., 2017), post-phase inactivation should only effect consolidation of training day processes. Moreover, contrary to our original hypothesis, we also now predict that mPFC-vHPC but not mPFC-dHPC pharmacological disconnection during either of these phases would impair the CPFE, as our results strongly suggest that communication between these structures is critical for these processes. In line with this, our results suggest that prefrontal communication with the vHPC occurs via the NR, whereas vHPC communication with mPFC occurs via direct projection (see **Chapter 6**). Therefore, pathway-specific silencing of direct mPFC-VMT, VMT-vHPC, or vHPC-mPFC projections during context preexposure will disrupt the CPFE. In our rat model of FASD, our results suggest that neonatal alcohol exposure disrupts prefrontal function that may be important for the formation of a detailed conjunctive context representation. Taken together with Murawski et al. (2012), these results suggest that this exposure targets neurobiology underlying both the acquisition and consolidation of incidentally-encoded contextual information. Despite not observing robust vHPC IEG expression after alcohol or sham intubation procedures (relative to undisturbed rats in Experiments 6.1 and 6.2), this effect on prefrontal function likely has adverse consequences on vHPC signaling, via the NR (which is heavily affected by PD4-9 alcohol exposure; see Gursky et al., 2019). An important test of this hypothesis is to use gain-of-function manipulations such as optogenetic excitation of specific prefrontal circuitry with the HPC (e.g., mPFC-NR, NR-mPFC, NR-vHPC) in alcohol-exposed rats. Whether impairments in similar thalamocortical pathways are seen in

humans with FASD remains to be seen, and is also a fruitful direction for future research.

## 9.4 Summary Statement

The goals of this dissertation were to elucidate prefrontal mechanisms underlying contextual learning and memory and how these processes are disrupted by third-trimester equivalent alcohol exposure in rats. This dissertation also examined the therapeutic potential of acute enhancement of cholinergic function in rescuing these deficits. As reviewed above, the results of this dissertation have several broad implications. First, we demonstrate a novel role of prefrontal and ventral hippocampal circuitry in incidental context learning and memory. By leveraging these discoveries, this work allows us to propose the neural circuitry underlying context and contextual fear learning and memory in the CPFE. This is important because processes of context and contextual fear are dissociated in this task, so this work allows for elucidation of the fundamental neural circuitry underlying these distinct processes in normally and abnormally developing (e.g., alcohol-exposed) animals. Second, we demonstrate the efficacy of acute physostigmine treatment in adolescence in reversing cognitive impairments seen in a rodent model of FASD. Taken together with our other studies (Dokovna et al., 2013; Robinson-Drummer et al., 2016, 2017), this work strongly suggest impaired cholinergic function within the prefrontal cortex and hippocampus plays an important causal role in these cognitive deficits. These studies provide a foundation for future work examining the efficacy of similar treatments in other models of FASD and in humans, and suggest a potential neural mechanism underlying the intervention.

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# Appendix A

## PERMISSIONS AND APPROVALS

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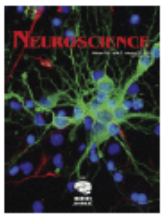


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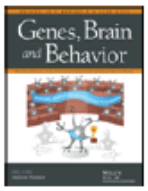


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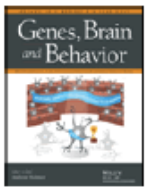


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| **Title** | Prefrontal Mechanisms of Impaired Cognition in a Rat Model of FASD | **Institution name** | University of Delaware |
| | | **Expected presentation date** | 2020-01-02 |
| **Instructor name** | Nicholas A. Heroux | | |

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| **Editor of portion(s)** | Nicholas A. Heroux | **Author of portion(s)** | Nicholas A. Heroux |
| **Volume of serial or monograph** | N/A | **Publication date of portion** | 1978-01-01 |
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## Appendix B

## IACUC Approval

**University of Delaware**
**Institutional Animal Care and Use Committee**
**Application to Use Animals in Research**
**(New and 3-Yr submission)**

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|---|---|
| **Title of Protocol:** Rodent Models of Cognitive Development: Eyeblink and Fear Conditioning and T-maze Learning | |
| **AUP Number:** 1104-2017-0 | ← **(4 digits only — if new, leave blank)** |
| **Principal Investigator:** Mark E. Stanton, Ph.D | |
| **Common Name (Strain/Breed if Appropriate):** Rat<br>**Genus Species:** *Rattus norvegicus* | |
| **Date of Submission:** 4/7/2017 | |

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IACUC Approval Signature: Steven Talbot, DVM

Date of Approval: 6/1/17