ONTOGENY OF CONTEXTUAL FEAR CONDITIONING: BEHAVIORAL, NEUROPHARMACOLOGICAL AND MOLECULAR MECHANISMS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology

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DEDICATION

For my family and friends and their ceaseless prayers and faith. Your love and hope carried me through this journey.

For my husband, I love you more today than ever before.

For Aunt Connie and her sweet potato pie.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
AC	anterior cingulate cortex
AP	anteroposterior
APV	2-amino-5-phosphonovaleric acid
Arc	activity-regulated cytoskeleton-associated protein
BAC	blood alcohol concentration
BAn	basal nucleus of the amygdala
BLA	basolateral complex of the amygdala
bp	base pair
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
cDNA	complementary deoxyribonucleic acid
CEn	central nucleus of the amygdala
cm	centimeter
CPFE	Context Preexposure Facilitation Effect
CR	conditioned response
CS	conditioned stimulus
dHPC	dorsal hippocampus
dL	deciliter
DPM	disintegrations per minute
DV	dorsoventral
EB	embryonic day
Egr-1	early growth response gene 1
EtOH	alcohol
FASD	Fetal Alcohol Spectrum Disorder
g	gram(s)
GABA	gamma-aminobutyric acid
GD	gestational day
H. M.	Henry Gustav Molaison
HC	homecage
HPC	hippocampus
hr	hour(s)
IEG	Immediate early gene
IL	infralimbic cortex
ISD	immediate shock deficit
kg	kilogram(s)
LA	lateral nucleus of the amygdala
mA	milliamp(s)

mAChr	muscarinic-type acetylcholine receptor
MD	mediodorsal thalamus
ME	multiple exposure CPFE protocol
mg	milligram(s)
min	minute(s)
mL	milliliter
ML	mediolateral
mm	millimeter
MMST	Multiple Memory Systems Theory
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
n	volume
nCi	nanocurie
NMDA	*N*-methyl-D-aspartate
NS	not significant
NVP	NVP-AAM077
PAG	periaqueductal gray
PBS	phosphate buffered saline
PD	postnatal day
PFC	orbitofrontal cortex
PL	prelimbic cortex
PNN	perineuronal net
RNA	ribonucleic acid
s	second(s)
sCFC	standard contextual fear conditioning
SE	single exposure CPFE protocol
Scop	Scopolamine
SI	sham intubated
UND	undisturbed
US	unconditioned stimulus
UTP	uridine-5'-triphosphate
vlPAG	ventrolateral periaqueductal grey
µg	microgram
µL	microliter

ABSTRACT

This dissertation characterizes learning-related molecular changes in developing rodents using a variant of contextual fear conditioning called the context preexposure facilitation effect (CPFE). The CPFE is a variant of contextual fear conditioning in which acquisition of the contextual representation (preexposure phase) and association of the retrieved contextual memory with an immediate foot-shock (training phase) are separated by 24 hrs. When tested 24 hr later, animals exhibit significant fear responding to the training context relative to control animals preexposed to an alternate context (Fanselow, 1986, 1990). During the CPFE, expression patterns of the inducible transcription factor early growth response-1 gene (*Egr-1*) vary based on training phase and brain sub-region in adult and adolescent rats (Asok, Schreiber, Jablonski, Rosen, & Stanton, 2013; Chakraborty, Asok, Stanton, & Rosen, 2016; Schreiber, Asok, Jablonski, Rosen, & Stanton, 2014). Importantly, regions in medial prefrontal cortex (mPFC) show a learning-related increase in *Egr-1* expression on the training day, whereby immediate shock increases expression in context-preexposed rats that learn fear, relative to control groups that do not. Behavioral expression of the CPFE emerges between the infant [postnatal day (PD) 17] and juvenile (PD 24) period in the rat (Schiffino et al., 2012; Robinson-Drummer & Stanton, 2015), but it is not known whether *Egr-1* expression correlates with this effect. Experiment 1 of this dissertation addressed this question by examining behavior and *Egr-1* expression in infant (PD 17) and juvenile (PD 24) rats during the CPFE using preexposure protocols involving a single-exposure or multiple-exposures to the training context. Following a single, 5 min preexposure to the training context, *Egr-1* expression in the mPFC, dorsal hippocampus (dHPC) and lateral nucleus of the

amygdala (LA) was increased in PD 24 rats but not PD 17 rats. In contrast, increased *Egr-1* expression following an immediate foot-shock (1 s, 1.5 mA) did not differ between PD 17 and PD 24 rats, and was not learning-related. Interestingly, increasing the number of exposures to the training chamber on the preexposure day (i.e. multiple-exposure protocol) altered training-day expression such that a learning-related increase in expression was observed in the mPFC in PD 31 and 24 but not PD 17 rats. However, across all experiments, behavioral expression of the CPFE was related to age-differences in *Egr-1* expression on the preexposure day but not the training day.

The second experiment in this dissertation used a rodent model of fetal alcohol spectrum disorder (FASD) to examine the effect of neonatal alcohol exposure on *Egr-1* mRNA expression following the training day of the CPFE. Prenatal alcohol exposure in humans can adversely affect postnatal cognitive, behavioral and neural development (Del Campo & Jones, 2017; Senturias & Asamoah, 2014). Extensive human and animal research on FASD has been invaluable in elucidating the neurobiological substrates of the disorder with key regions like the hippocampus and frontal cortex being particularly susceptible to the teratogenic effects of early alcohol exposure (Fryer et al., 2007; Lebel, Roussotte, & Sowell, 2011; Norman, Crocker, Mattson, & Riley, 2009). The CPFE is a hippocampus-dependent task (G. F. Hamilton et al., 2011; Matus-Amat, Higgins, & Rudy, 2004; Matus-Amat, Higgins, Sprunger, Wright-Hardesty, & Rudy, 2007; Robinson-Drummer, Dokovna, Heroux, & Stanton, 2016) that is also disrupted by neonatal alcohol exposure in adolescent rats (Dokovna, Jablonski, & Stanton, 2013; Jablonski & Stanton, 2014; Murawski & Stanton, 2010, 2011). Experiment 2 characterized *Egr-1* expression following the training day in animals preexposed to the training context (Group Pre) and non-associative control

animals preexposed to an alternate context (Group Alt-pre). Rat pups were exposed to a 5.25 g/kg/day single binge-like dose of alcohol (Group EtOH) or were sham intubated (Group SI) over postnatal days PD 7-9. Animals exposed to alcohol failed to show the CPFE replicating our previous findings (Jablonski & Stanton, 2014; Murawski & Stanton, 2011). Furthermore, alcohol exposure reduced cortical *Egr-1* expression following training such that the learning–related increase in *Egr-1* observed in the mPFC of SI animals was abolished in Group EtOH. However, decreases were not observed in the lateral nucleus of the amygdala or the hippocampus relative to Group SI.

The previous results suggest that regional molecular activity in the mPFC is a better indicator of CPFE performance than the hippocampus or amygdala. The mPFC plays a critical role in contextual fear conditioning (Corcoran & Quirk, 2007; Heroux, Robinson-Drummer, Sanders, Rosen, & Stanton, 2017; Zelikowsky et al., 2013) and develops abnormally following neonatal alcohol exposure (G. F. Hamilton, Whitcher, & Klintsova, 2010c; Whitcher & Klintsova, 2008). Additionally, impaired cholinergic function contributes to the learning impairment observed in the CPFE following alcohol exposure (Dokovna et al., 2013). The final experiment examined the specific contribution of the mPFC cholinergic system to each phase of the CPFE in normal adolescent rats (PD 31). Adolescent rats received bilateral infusions of scopolamine (35μg/side) or phosphate buffered saline (PBS) 10 min before all three phases of the CPFE or only prior to a single phase. Intra-mPFC administration of scopolamine prior to all three phases significantly impaired fear conditioning suggesting that mPFC cholinergic function is necessary for successful CPFE performance. Analyses of the individual infusion days revealed a significant impairment of the CPFE when

infusions occurred prior to preexposure or training (i.e. immediate foot-shock) but not prior to testing

These studies demonstrate a clear maturation of *Egr-1* expression between PD 17 and PD 24 that is both age- and experience-dependent. The data suggest that regional activity and plasticity within the mPFC on the preexposure day may contribute to impaired context representation processes in infant rats. In addition, the diminished mPFC *Egr-1* expression observed following neonatal alcohol exposure on the training day likely reflects impaired memory of the context-shock association. Finally, cholinergic function in the mPFC is critical for successful performance of the CPFE and suggests a potential mechanism by which neonatal alcohol exposure may impair learning or memory in this task. Taken as a whole, this dissertation reveals the versatility of the CPFE in identifying the neurological correlates and mechanisms of contextual learning in both normal and abnormal development.

Chapter 1

INTRODUCTION

1.1 Goals of Dissertation

This dissertation seeks to provide novel insights into the molecular processes by which memory capabilities mature over ontogeny and to propose a mechanism by which cognitive development can be disrupted following neonatal brain injury. Particularly, this dissertation utilizes a variant of Pavlovian fear conditioning to characterize developmental changes in the neocortical mechanisms of contextual conditioning. It also demonstrates the role these mechanisms may play in cognitive impairment observed in a rodent model of Fetal Alcohol Spectrum disorder (FASD; see Chapter 4). Finally, this dissertation will draw parallels between deficits observed in FASD rats and those observed following localized drug infusions into medial prefrontal cortex in order to propose a neurochemical mechanism by which neonatal alcohol exposure produces learning deficits.

1.2 The Prefrontal Cortex and Contextual Fear Conditioning

The neurobiological circuit necessary for contextual fear learning (Chapter 2), which originally emphasized the importance of the hippocampus and the amygdala (Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001; Phillips & LeDoux, 1992), has been expanded to include the medial prefrontal cortex (mPFC; Giustino & Maren, 2015; Maren, Phan, & Liberzon, 2013; Rozeske, Valerio, Chaudun, & Herry, 2015). During contextual fear learning, the mPFC likely participates in the acquisition of the

context representation in conjunction with the hippocampus. Electrophysiological reports have shown context-specific activity in the prefrontal cortex (Hyman, Ma, Balaguer-Ballester, Durstewitz, & Seamans, 2012) as well as prefrontal modulation of hippocampal spatial representations (Kyd & Bilkey, 2003, 2005). Both regions increase their activity during acquisition of contextual fear conditioning (Asok et al., 2013; Chakraborty et al., 2016; Zelikowsky, Hersman, Chawla, Barnes, & Fanselow, 2014) and the mPFC is capable of supporting fear conditioning when the hippocampus is lesioned (Zelikowsky et al., 2013). What these studies reveal is a contribution of the mPFC to the initial acquisition of contextual memories that may be just as crucial to conditioning as that provided by the hippocampus. The current dissertation will extend our knowledge of mPFC involvement in contextual fear conditioning by examining prefrontal function during conditioning in normally developing rats and in rats neonatally exposed to alcohol, a rodent model of FASD.

1.3 The CPFE Paradigm

This dissertation utilized as its behavioral paradigm the context preexposure facilitation effect (CPFE; Figure 1.1), a variant of the standard contextual fear conditioning (sCFC). In the sCFC protocol, animals are placed into a training chamber and allowed to freely explore the environment for about 2 min and then an aversive stimulus (i.e. foot-shock) is delivered through the chamber floor. Using this protocol, acquisition of the context representation and association of that representation with the aversive foot-shock happen in the same conditioning trial. In contrast, during the CPFE, acquisition of the context representation (preexposure phase) and association of the context representation with an aversive stimulus (training phase) occur on separate, successive occasions (Fanselow, 1986, 1990). During preexposure, animals

are exposed to the training context (Context A; Group Pre) for 2-5 min without foot-shock and are then returned to their home cages. A second set of animals, which serve as non-associative controls, are exposed to an alternate context (Context B; Group Alt-Pre) for the same duration as Group Pre. Twenty-four hours later during training, both groups Pre and Alt-Pre are given an immediate foot-shock in Context A. The acquisition of contextual fear memory is typically quantified in terms of time spent freezing upon re-exposure to training context during testing, as assessed immediately (using a post-shock freezing test) or after a retention interval (typically 24 hr). Group Pre shows elevated freezing relative to both Group Alt-Pre, which displays the immediate-shock deficit (ISD), and a no-shock control group (Fanselow, 1990).

The CPFE has a protracted rate of development in the rat (Chapter 3). Pre-weanling rats at PD 17 or PD 19 fail to show conditioned freezing to the training context, whereas between PD 21 and PD 24 the effect emerges without further development during adolescence or adulthood (Jablonski, Schiffino, & Stanton, 2012; Robinson-Drummer & Stanton, 2015; Schiffino, Murawski, Rosen, & Stanton, 2011). It is likely that neurobiological development of key regions in the fear circuit contribute to this developmental dissociation (Stanton, 2000) so the first question examined in this dissertation is, *will infant amygdala, hippocampal or medial prefrontal gene expression changes differ from older animals following context preexposure and immediate shock training?* With the known contributions of the immediate early gene called early growth response-1 (*Egr-1*) to long-term memory processes (see Alberini, 2009, 2011), impairments in its induction may result in impaired consolidation of context-learning and/or the context-shock association,

leading to a failure in conditioned freezing during the CPFE. These results are presented in Chapter 7.

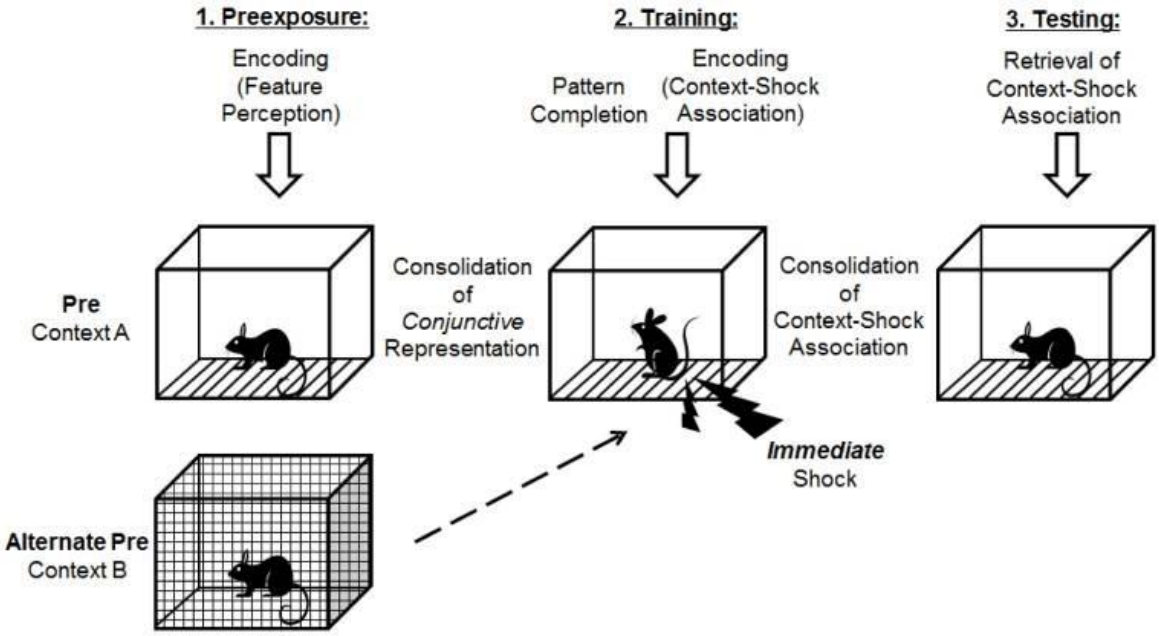

Figure 1.1 Schematic representation of the Context Preexposure Facilitation Effect (CPFE) and associated memory processes. During the first phase of training ('Preexposure') rats are placed into the novel training context designated Context A (Group Pre) and allowed to freely explore while a second group is exposed to an alternate context designated Context B (Group Alt-Pre). During preexposure, the individual spatial/contextual features of the context are encoded for subsequent consolidation into a single conjunctive representation of the context. Twenty-four hours later ('Training'), both groups Pre and Alt-pre are placed into the training context (Context A) and an immediate (< 5 s) foot-shock is delivered. In the seconds prior to foot-shock delivery, a subset of the context features is able to elicit retrieval of the previously acquired conjunctive context representation in Group Pre through pattern completion but not Group Alt-pre. Foot-shock is associated with the retrieved context representation and the context-shock association is subsequently consolidated. Twenty-four hours later ('Testing'), all rats are returned to the training context (Context A) and tested for fear of the training context (i.e. species-typical freezing behavior). Animals preexposed to the training context (Group Pre) retrieve the context representation memory associated with foot-shock and display increased freezing to the context relative to group Alt-Pre (image taken from Jablonski and Stanton, 2014).

1.4 The CPFE and Fetal Alcohol Spectrum Disorder (FASD)

The CPFE has been used to investigate learning-related changes in molecular function during context learning and context-shock association. We previously demonstrated, in both adolescent (PD 31) and adult rodents, following immediate foot-shock training, that mPFC *Egr-1* levels in animals that learn the CPFE are significantly elevated above non-associative control animals that are preexposed to an alternate context (Chapter 5; Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014). The importance of *Egr-1* activity for contextual fear conditioning has been demonstrated in adult rats with reduction of amygdalar or hippocampal *Egr-1* levels significantly impairing contextual fear conditioning (J. L. Lee, 2010; Malkani & Rosen, 2001; Malkani, Wallace, Donley, & Rosen, 2004). The second question in this dissertation is, *how does neonatal alcohol exposure change patterns of Egr-1 expression in the amygdala, hippocampus and prefrontal cortex following immediate shock training?* Alcohol exposure from PD 7-9 impairs fear conditioning when assessed 24 hr after training, however immediate post-shock freezing is preserved suggesting a failure of long-term consolidation of the context-shock association in these animals (Chapter 4). This likely will be reflected in diminished *Egr-1* expression in FASD rats relative to sham-intubated controls following the training phase of the CPFE. These results are presented in Chapter 8.

The significant impairment in CPFE performance observed following cholinergic antagonism (Robinson-Drummer et al., 2016) is relevant to behavioral impairments in FASD (Chapter 4). Rodent models of FASD reveal a cholinergic contribution to alcohol-related learning impairments (Hunt, 2012; Hunt, Barnet, Shea, & Baker, 2006; Ryan, Williams, & Thomas, 2008; Wagner & Hunt, 2006). The CPFE is disrupted by neonatal alcohol exposure (G. F. Hamilton et al., 2011; Jablonski &

Stanton, 2014; Murawski & Stanton, 2010, 2011) and this impairment is rescued by enhancement of cholinergic function (Dokovna et al., 2013). Subsequently, several reports have shown that alcohol-induced behavioral and molecular changes in the fear circuit (particularly in the prefrontal cortex; see Chapter 2) can be rescued with choline supplementation (Monk, Leslie, & Thomas, 2012; Otero, Thomas, Saski, Xia, & Kelly, 2012; Schneider & Thomas, 2016; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007; Thomas, Idrus, Monk, & Dominguez, 2010; Thomas & Tran, 2012). The final question asked in this dissertation is, *how does prefrontal cholinergic function contribute to contextual fear conditioning in the CPFE?* This questions is addressed by pharmacologically antagonizing cholinergic function in the prefrontal cortex prior to context learning, context-shock association or fear memory testing during the CPFE. These results are presented in Chapter 9.

1.5 Dissertation Outline

This dissertation is organized into three sections. In the first section (Chapters 2-5), relevant background information is provided including the neurobiology of contextual fear conditioning (Chapter 2), the ontogeny of learning and memory (Chapter 3) and FASD and cognitive function (Chapter 4). Chapter 5 presents previously published work that was foundational for this dissertation. The second section begins with a description of the general methods used in all experiments (Chapter 6) followed by detailed explanation and analysis of each dissertation project (Chapter 7-9). Finally, the third section includes discussion of the dissertation questions, conclusions and future directions (Chapter 10).

Chapter 2

NEUROBIOLOGY OF CONTEXTUAL FEAR CONDITIONING

This thesis focuses on the CPFE (Chapter 1, Figure 1.1). However, much of what is known about the neurobiology of contextual fear is based on standard contextual fear conditioning (sCFC). Typically, sCFC involves a context-shock association that can be acquired when the context is the sole predictor of the US (foreground context conditioning) or if a discrete cue (e.g., a tone) is being paired with the US (background context conditioning; Fanselow, 2010; Nadel & Willner, 1980; Rudy, 2009). Although an exhaustive discussion is beyond the scope of this dissertation, a brief exposition on the neurobiology of context conditioning is necessary to establish a foundation for the research described in the coming sections.

2.1 Theoretical Perspectives Concerning Contextual Memory

Following the bilateral temporal lobectomy of patient H. M., research into the neural basis of learning and memory culminated in an early psychological theory known as the multiple memory systems theory (MMST; Squire, 1992). This theory postulated separate and distinct neurological systems for different types of learning and memory and the hippocampus was identified as critical for context learning. However, animal research showing that conditioning to contextual stimuli requires the hippocampus only under certain conditions revealed conflicting evidence for the MMST (Matus-Amat et al., 2004; Rudy, Barrientos, & O'Reilly, 2002; Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006). Subsequently, the dual-process theory of context representations provided a unique framework for reconciling these disparate findings. This theory proposed that context memories can be comprised of either slowly-acquired, elemental features, supported by a hippocampus-independent

neocortical system, or a rapidly-acquired conjunctive representation, made of the individual context elements bound together, that depends on hippocampus (Maren, 2001; Nadel & Willner, 1980; O'Reilly & Rudy, 2001; Rudy, 2009; Rudy, Huff, & Matus-Amat, 2004). Behavioral evidence in support of this theory has shown that sCFC, which can be supported by the elemental system, can be acquired without a hippocampus (Wiltgen et al., 2006; Zelikowsky, Bissiere, & Fanselow, 2012). However the CPFE, which requires utilization of a conjunctive representation is impaired when the hippocampus is not functional and, most importantly, cannot be supported by preexposure to the individual features of the training context (Matus-Amat et al., 2004; Robinson-Drummer et al., 2016; Rudy et al., 2002; Rudy & O'Reilly, 1999; Schiffino et al., 2011). Neurobiological evidence for dual-process context learning is discussed in the next section. Further discussion of behavioral evidence is discussed at other points in this thesis (Sections 2.3 and 2.5).

2.2 Neuroanatomical Basis for Contextual Fear Conditioning

The neuroanatomical circuitry specific to contextual fear conditioning has largely been determined over the last 25 years (Fanselow, 2010; Fendt & Fanselow, 1999; LeDoux, 2000; Marek, Strobel, Bredy, & Sah, 2013; Maren, 2001; Maren et al., 2013; Phillips & LeDoux, 1992; Sotres-Bayon & Quirk, 2010). In an early model, Fendt and Fanselow (1999) detailed the likely pathway mediating sCFC (Figure 2.1). Information about both the contextual CS and the aversive US travel to the thalamus. Contextual information is projected from the thalamus and further processed through the parahippocampal gyrus to the hippocampus (HPC) where it then projects to the amygdala. This contextual information, they predicted, converges with the US information on the lateral (LAn) and basal (BAn) nuclei in the basolateral complex

(BLA) of the amygdala which communicates bidirectionally with the central nucleus (CEn) of the amygdala. Efferent projections from the CEn of the amygdala to midbrain and brainstem nuclei activate motor regions responsible for specific forms of conditioned responding (e.g. freezing, potentiated startle, bradycardia, etc.) resulting in the behavioral "read out" of fear conditioning.

More recently, details regarding the roles of the specific brain regions have supported and extended much of the original modeling with the addition of new brain areas and new roles for previously proposed areas. For instance, while Fendt and Fanselow (1999) ascribed mostly output functions to midbrain structures like the periaqueductal gray (PAG), Herry and Johansen (2014) described a potential role of the PAG not simply as a CEn efferent motor region for the expression of fear memory but also as a potential locus of fear learning. This region receives ascending nociceptive information from the spinal and trigeminal dorsal horn that is relayed to the amygdala, possibly, through indirect pathways by way of midline thalamic nuclei or dorsal medial prefrontal (mPFC) cortices. This projection circuit may serve instructive purposes relaying US information to the amygdala in order to facilitate fear learning. The mPFC was not included in early contextual fear circuit models (Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001) however growing reports suggest several functions of the mPFC during contextual fear conditioning (Giustino & Maren, 2015; Marek et al., 2013; Quirk & Beer, 2006; Rozeske et al., 2015; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Sotres-Bayon & Quirk, 2010; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006).

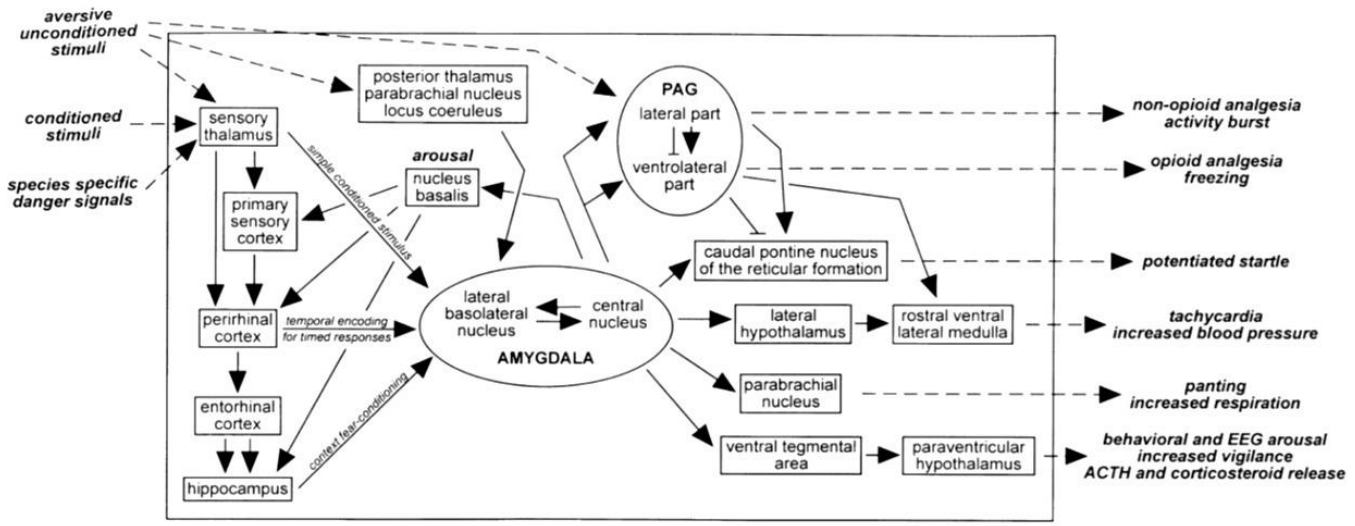

Figure 2.1 Hypothetical circuit proposed by Fendt and Fanselow (1999) to mediate the behavioral and neurological components of conditioned fear. Much of the circuitry has been confirmed however the prefrontal cortex, which was not implicated in conditioned fear originally, is not featured. Figure taken from Fendt and Fanselow (1999).

2.3 The Hippocampus: Critical for Context Learning?

As mentioned previously (Section 2.1), the hippocampus' primary role during contextual fear conditioning is to create a representation of the training context. Hippocampal place cells, which are responsive to both spatial and non-spatial (i.e. contextual) environmental stimuli, fire differentially between contexts and also show re-mapping of their firing fields when the contextual components of an enclosure are changed (M. I. Anderson & Jeffery, 2003; Deshmukh & Knierim, 2013; Maren et al., 2013; Moser, Kropff, & Moser, 2008; O'Keefe & Nadel, 1978; Smith & Mizumori, 2006). However, there is also evidence that the hippocampus receives and manipulates US information during fear conditioning. Lovett-Barron et al. (2014) showed that hippocampal interneurons, which are activated by entorhinal input during the US onset, inhibit concurrent activation of HPC pyramidal cells during the US. This they suggest compartmentalizes the associations between the CS and the US to the amygdala and ensures that the contextual CS alone will elicit conditioned responding in the absence of the US. Furthermore, disinhibition significantly impaired contextual fear conditioning supporting the functional roll of inhibiting incoming entorhinal activity. Together, these reports suggest that an individual context representation within the hippocampus is established by the population coding of these cells and maintained through inhibitory activity with the entorhinal cortex which acts to stabilize the context representation during conditioning.

There are circumstances under which compensatory mechanisms are capable of supporting contextual fear conditioning in the absence of the hippocampus. The dual-process theory of context representations (Section 2.1, above) suggests that a secondary cortical system, including neocortical regions in the parahippocampal

gyrus, is typically suppressed by hippocampal activity during training and is disinhibited when the hippocampus is damaged allowing this system to mediate contextual learning (Nadel & Willner, 1980; Rudy et al., 2002; Rudy et al., 2004). The hippocampus is thought to form a conjunctive representation of the environment in which an event takes place and to be necessary for the initial encoding of contextual information for subsequent consolidation and storage. However, these roles are challenged by reports that show with sufficient training and/or time between exposure to the context and conditioning, sCFC can be achieved with pre-conditioning hippocampal lesions (Ballesteros, de Oliveira Galvao, Maisonette, & Landeira-Fernandez, 2014; J. Q. Lee, Sutherland, & McDonald, 2017; Matus-Amat et al., 2004; Wiltgen et al., 2006; Zelikowsky et al., 2012). In the rodent, the perirhinal, postrhinal and entorhinal cortices are suggested to support contextual learning under these circumstances (for review see Rudy, 2009) however, the characteristics of the conditioning are different. More training is required for hippocampus-lesioned animals to reach similar levels of conditioned responding as intact animals (Wiltgen et al., 2006; Zelikowsky et al., 2012) and context generalization and rates of forgetting increase indicating that the secondary system may establish a cruder and possibly less stable representation of the context (Wiltgen & Tanaka, 2013; Zelikowsky et al., 2012). These counterexamples, however, do not disqualify the hippocampus from being the primary region responsible for context learning under normal circumstances in intact animals.

2.4 **The Prefrontal Cortex: A New Player in an Old System**

The previous section (Section 2.3) highlighted the importance of the hippocampus to contextual conditioning however a growing body of work suggests

that the hippocampus and the mPFC work in concert to acquire contextual fear memories. Rozeske et al. (2015; Figure 2.2) proposed that dorsal mPFC regions receive inputs from the ventral CA1 and project to the BLA where they possibly modulate contextual fear acquisition. Output circuits that include the dorsal mPFC and amygdala project to the ventrolateral periaqueductal grey (vlPAG) to produce conditioned fear responses. Variations in connectivity along its dorso-ventral axis have revealed functional differentiation of mPFC subregions (Heidbreder & Groenewegen, 2003) and variations in interconnectivity with sensory (thalamus), spatial (hippocampus and parahippocampal gyrus) and emotional (amygdala) systems suggest that the mPFC participates in contextual fear conditioning through sub-region specific interactions (Hoover & Vertes, 2007).

Behavioral and molecular findings support the complementary HPC-mPFC relationship during contextual fear acquisition suggested by neuroanatomical results summarized in Figure 2.2. During sCFC, animals placed into a training context and shocked after a delay show increased cellular activity [as measured by activity-regulated cytoskeleton-associated protein (*Arc*) expression] in the dorsal HPC and prelimbic region (PL) of mPFC (Zelikowsky et al., 2014). Interestingly, examination of cells active during conditioning reveal that a significant number of these cells are also active during memory retrieval while very few cells were active during training or testing alone. This effect was strong in both regions and suggests that both regions process context-specific information. Interestingly, however, the response to the training context following both training and testing was evident regardless of whether a context-shock association was acquired. Animals who were placed in the training context and given an immediate shock did not learn an association between the context

and foot-shock but had similar cellular activity as the associative learning group. This suggests that both regions are responding to the spatial-contextual information and not the pairing of the context with shock. In contrast, the amygdala differentially responded to the training versus memory test in a learning-related way with activity within a neuron following both experiences only evident in the associative learning group. This supports the role of the amygdala as ultimately processing the context-shock association while the spatial-contextual information is provided by both the HPC and mPFC.

In the absence of the hippocampus, reports suggest that acquisition of fear memories is carried out by the mPFC. Zelikowsky et al. (2013) lesioned the dHPC, mPFC [either the Infralimbic (IL) or Prelimbic (PL) regions] or both regions prior to background contextual fear conditioning. During retention testing in the training context, animals with a single lesion (either the dHPC or in the mPFC) were able to retrieve the fear memory acquired 24 hr prior. Similarly, pharmacological inactivation of the mPFC alone prior to either foreground or background sCFC also spared fear memory (Corcoran & Quirk, 2007; Heroux et al., 2017). However, for animals with combined hippocampal-mPFC lesions (dHPC and IL or dHPC and PL), fear memory was significantly disrupted (Zelikowsky et al., 2013). The effect of the double lesion was only observed when lesions were made in contralateral hemispheres, resulting in a functional disconnection of these regions, suggesting that these two regions communicate during the acquisition of contextual fear. On the other hand, other studies that have examined prefrontal involvement in context fear memory have yielded conflicting results. In contrast to Zelikowsky et al. (2013), reversible, pharmacological inactivation of the PL alone (Gilmartin & Helmstetter, 2010;

Gilmartin, Kwapis, & Helmstetter, 2013) using Muscimol ($GABA_A$-receptor agonist), APV or NVP (NMDA-type glutamate receptor antagonists) caused significantly reduced memory of background contextual fear conditioning when animals were tested drug-free. Additionally, Corcoran and Quirk (2007) showed impaired fear acquisition, as measured by post-shock freezing during conditioning, in mPFC animals while Heroux et al. (2017) found no effect of mPFC inactivation on post-shock freezing. Across these reports both the number of training trials increased and the contextual exposure time increased so whether either or both of these factors contributed to the conflicting results will need to be determined experimentally.

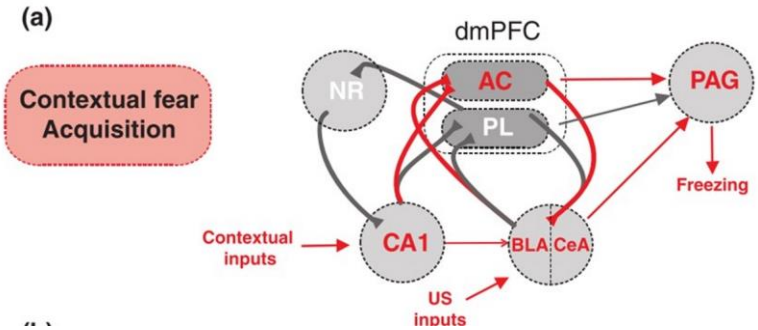

Figure 2.2 Updated working model of neuronal circuits that mediate contextual fear memory acquisition and expression. Contextual fear acquisition requires a contextual representation of the environment within the hippocampal CA1 to be relayed to the basolateral and central nuclei of the amygdala (BLA/CeA), for context-US association. Dorsal medial prefrontal (dmPFC) regions, receives inputs from the ventral CA1 and projects to the BLA where they likely contribute to modulation of contextual fear acquisition. Image adapted from Rozeske et al. (2015).

2.5 Spatial Representations and the Medial Prefrontal Cortex

The mPFC may contribute to contextual learning through modulatory control over hippocampal spatial activity (Hok, Chah, Save, & Poucet, 2013; Kyd & Bilkey, 2003, 2005). Kyd and Bilkey (2003) lesioned the mPFC and recorded from hippocampal place cells in rats foraging in an open field. Overall, place cell information content (bits/spike) increased in the lesioned animals relative to shams suggesting that spatial localization may be stronger in this group. Also, the center of mass of place fields, which was stable within a recording session, shifted more in lesioned animals than in shams after reexposure to the same environment following a 3 min delay. An even larger shift in place fields was observed after a 5 hr delay between exposures. This increased shift in the lesioned groups indicates that the mPFC usually imposes an inhibitory effect on hippocampal spatial processing and helps to enhance stability of the larger contextual environment by reducing irrelevant information. In addition, HPC place cells were significantly influenced by local cues; placing objects in the test context disrupted place fields significantly more in lesioned animals relative to sham controls (Kyd & Bilkey, 2005). The increase in local cue sensitivity and the instability across time imply that the mPFC adds a stabilizing influence on hippocampus-dependent context representations both during encoding and across time (e.g. during consolidation periods).

The mPFC may also possess its own context-specific (place-cell-like) activity. Hyman et al. (2012) showed that single-unit activity recorded during exposure to two distinct environments (Context A vs Context B) revealed preferential firing in a small but significant population of mPFC neurons similar to that of hippocampal place-cell firing. When comparing population firing with respect to distinct environments, the contextual information being encoded by the mPFC was qualitatively different from

that being encoded by hippocampus. While hippocampal ensembles differentiated between distinct locations within a single environment, mPFC ensembles differentiated strongly between the same location across different exposures. Furthermore, the authors note that some mPFC ensembles showed a systematic shift in activity to a given location during subsequent exposures which allowed the experimenters to accurately predict the network state of some ensembles upon subsequent exposure to the environment. The predictable shift in context-specific firing across time may allow the PFC to encode a time-stamped representation of a large-scale contextual environment that is consistent within a given environment in order to supplement the hippocampus' detailed spatial-contextual representation.

If the mPFC were a major contributor to the neocortical system for context learning (Section 2.1; Rudy, 2009) in the absence of the hippocampus, the aforementioned context-specific neuronal activity could facilitate a crude (i.e. low-detail, large scale) representation of the environment predicted by the dual-process theory of context learning (Nadel & Willner, 1980; Rudy, 2009). Rudy (2009) suggested that the contextual representations formed by the neocortical system were by necessity an elemental representation of the context. However, Fanselow (2010) counter–argued that if the context representations were elemental then contextual fear conditioning without the hippocampus should show an acquisition curve similar to that of cued conditioning (i.e. an inverse relationship between amount of cue preexposure and associability). Because context fear learning in the absence of the hippocampus still shows facilitation with preexposure, Fanselow (2010) suggested that the context representation constructed by the neocortical system may still be conjunctive. The previously mentioned evidence that the mPFC can modulate spatial

information, respond predictably to spatial (contextual) information and stabilize contextual information across time, suggests that the mPFC may be the region creating the “conjunctive-like” representation suggested by Fanselow (2010), with the parahippocampal regions. These results confirm a role of the mPFC to context learning however, whether or not the context representation acquired without the hippocampus is truly elemental or bound into a crude conjunctive representation is currently unknown.

2.6 **Conclusions**

The current chapter demonstrates the value of contextual fear conditioning as a means to delineate the neurological basis for learning and memory. As new evidence has emerged, the psychological theories of contextual fear conditioning have been updated to include roles for previously unacknowledged cortical regions. Previous theories which placed the hippocampus at the center of context learning must now reexamine this key position as one that may be complimented by the prefrontal cortex. With this in mind, the current dissertation will propose a role of the medial prefrontal cortex in the ontogeny of normal context learning and disruption of this role may contribute to impaired learning when prefrontal function is compromised.

Chapter 3

ONTOGENY OF LEARNING AND MEMORY

The study of learning and memory across ontogeny has revealed that some properties of context conditioning are developmentally dissociable. In conjunction with the previous section (Chapter 2), the current chapter will review learning and memory in developing animals in relation to neurobiological changes associated with the development of memory.

3.1 Infantile Amnesia: A Historical Perspective

One of the earliest discoveries in the ontogeny of memory is known as infantile amnesia. Infantile amnesia is characterized by a failure of adults to recall declarative, episodic memories that occurred during early childhood (< 5 years of age). This contrasts with memories established at later points in ontogeny that are easily retained over comparable or even longer periods of adulthood. In his essay, Freud (1910) identified the potential importance of studying the psychological basis for infantile amnesia in reference to treating adult mental disorders:

> "May there not be an ultimate connection between the infantile and the hysterical amnesia? The connection… is really more than a mere play of wit…We may say that without infantile amnesia there would be no hysterical amnesia. " (p. 37)

Nested in that inquiry, Freud proposed that childhood forgetfulness may be mechanistically homologous to adult memory deficits and that determining the basis for infantile amnesia may prove invaluable to the treatment of memory disorders in adulthood. More than one hundred years later, developmental psychobiologists echo Freud with the following statement (Callaghan, Li, & Richardson, 2014):

> "We may then begin to unravel whether the same mechanisms are involved in [infantile amnesia], spontaneous memory loss in adulthood, and memory loss in pathological aging … Furthermore, we hope that future studies on the process of [infantile amnesia] will help to elucidate the mechanisms involved in memory across the lifespan…" (p. 52)

Campbell and Campbell (1962) are credited with discovering that infantile amnesia can be demonstrated in a rodent model. They used an active avoidance task to assess acquisition and retention of learning in rats (Figure 3.1). Animals were trained on postnatal day (PD) 18, 23, 38, 54 or 100 to actively avoid the shock-paired side of a two-compartment chamber. These rats were then tested at a variety of retention intervals (ranging from immediately after conditioning to 42 days later) for responding in the chamber. As expected, all rats showed high levels of responding during the immediate test but there was a significant age by retention interval interaction. The youngest age (PD 18) displayed significantly reduced recall of the training experience after only 7 days. Conversely, memory retention increased significantly as a function of training age such that the older the age of the animal at training the longer the retention of avoidance responding thus indicating an age-dependent retention of learning. In subsequent decades, well controlled studies using human infants generated retention functions almost identical to those observed in rodent models. Rovee-Collier and colleagues (Greco, Rovee-Collier, Hayne, Griesler, & Earley, 1986; Hartshorn et al., 1998; Rovee-Collier, 1997; Vanderlinde, Morrongiello, & Rovee-collier, 1985), across several studies, used two tasks to track the memory retention of infants between two and 18 months old (Figure 3.2). In the first task (used on infants from 2-6 months), infants learned to move an over-hanging mobile by kicking their ankle which was tied to the mobile. In the second task (used on infants from 6-18 months), infants learned to move a small train around a circular track by depressing a lever. At the

youngest age examined, retention for the mobile task was less than a week although a linear relationship between age at training and retention in weeks was observed such that by 18 months, infants could display memory for the train task 13 weeks after initial training. These tasks in humans and the previously described tasks in rodents illustrate the increasing capacity of developing animals to retain previously acquired memory for longer periods as they age.

Today there is a wealth of information concerning infant learning and memory and its psychological determinants in both animal models (Akers et al., 2014; M. J. Anderson & Riccio, 2005; Campbell & Campbell, 1962; Campbell, Misanin, White, & Lytle, 1974; Campbell, Sananes, & Gaddy, 1984; Guskjolen, Josselyn, & Frankland, 2016; Richardson, Riccio, & Axiotis, 1986; Richardson, Riccio, & Jonke, 1983; Richardson, Riccio, & McKenney, 1988; Robinson-Drummer & Stanton, 2015) and humans (for review see Rovee-Collier, Hayne, & Colombo, 2000). Research using animal models has very recently begun to reveal the neural mechanisms of infantile amnesia, although much remains to be learned (Josselyn & Frankland, 2012; S. Li, Callaghan, & Richardson, 2014; Madsen & Kim, 2016). I now briefly review the mechanisms of infant memory development at psychological (Section 3.2) and neurobiological (Section 3.3) levels.

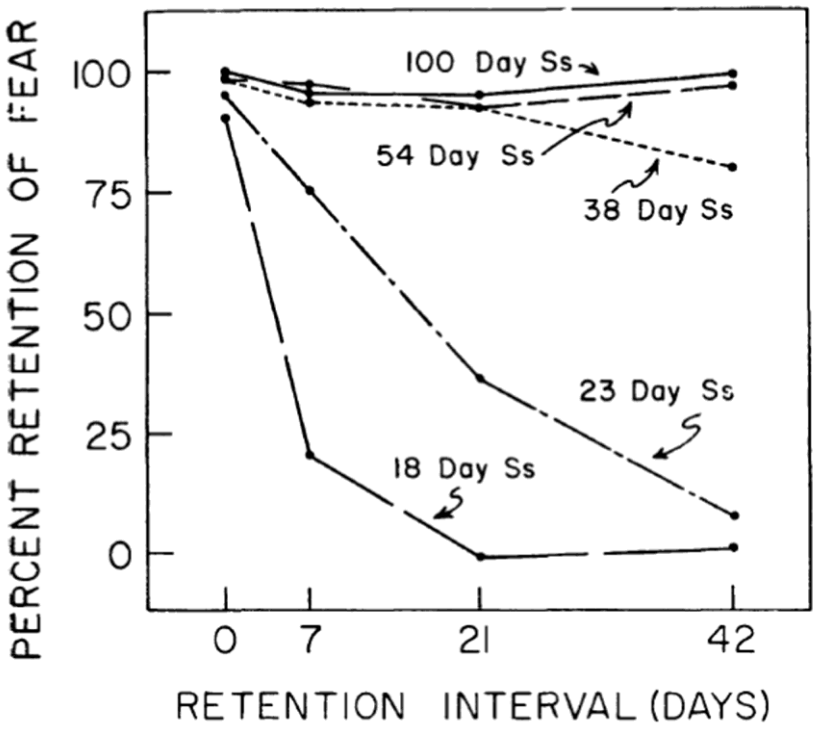

Figure 3.1 Mean percent retention of fear following active avoidance conditioning as a function of age 0, 7, 21, and 42 days after conditioning. Animals were trained to avoid the shock-paired side of a two-compartment fear chamber. Initial rates of learning were comparable between ages as indicated by the 0 day retention interval. However, animals trained at PD 18 displayed rapid forgetting of conditioning evident 7 day after training. Forgetting decreased with age with near perfect long-term retention of avoidance observed in the two oldest age groups. Image taken from Campbell and Campbell (1962).

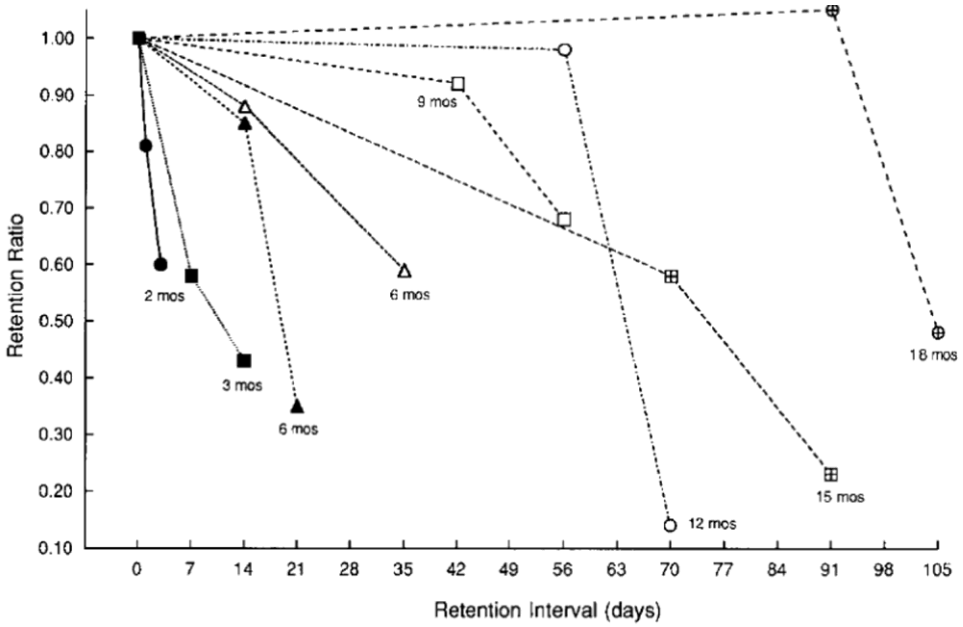

Figure 3.2 Mean retention ratio of operant conditioning in developing infants (2-18 months old at training) as a function of retention interval (0-105 day) after training. Infants were trained to kick their ankles in order to move an overhanging mobile (filled shapes) or press a lever to activate a toy train (open shapes). Points represent performance during the immediate retention test, after the longest test delay at which a group exhibited retention and after the longest delay tested at which a group exhibited no retention. Initial learning was equivalent across ages as indicated by similar ratios during the immediate test. Forgetting was on the order of days in infants trained at 2 or 3 months old. Memory retention increased with increased age at training. Image taken from Hartshorn, Rovee-Collier et al., 1998.

3.2 Psychological Mechanisms of Infant Memory Development

The psychological processes responsible for deficits in memory during development can be grouped into three categories: memory encoding, storage or retrieval (Josselyn & Frankland, 2012; S. Li et al., 2014). Of these three, encoding failure is the least likely to underlie memory deficits during development. Deficits in encoding would result in poorer levels of acquisition or initial memory expression between developing animals and adults. However, both human and animal studies have repeatedly demonstrated equivalent levels of acquisition during and following training (Figures 3.1 and 3.2; Akers, Arruda-Carvalho, Josselyn, & Frankland, 2012; Campbell & Campbell, 1962; Greco et al., 1986; Hartshorn et al., 1998). Furthermore, even following overtraining, infant rats still show forgetting at a rate much higher than older animals (Guskjolen et al., 2016). Based on these findings encoding failures are unlikely to be responsible for memory deficits in infants.

It is more likely that rapid forgetting following equivalent acquisition is either a storage or retrieval failure. A storage decrement account of infant forgetting posits that a memory trace decays over time until it is no longer stored. If so, reminder cues should be ineffective at reactivating the lost memory. In infant rats, spontaneous forgetting of cued fear conditioning, which is observed only a few days after acquisition, can be alleviated by a brief reminder treatment prior to testing (J. H. Kim & Richardson, 2007a, 2007b; S. Li, Kim, & Richardson, 2012b; Revillo, Cotella, Paglini, & Arias, 2015). Similarly, implicit memory tasks in which children are exposed to elements of a forgotten training episode also suggest memory traces are adequately stored but inaccessible. Children given a single prompt (i.e. exposure to the original mobile or train) 1 week after forgetting the mobile task, showed reactivation of the original memory (Hildreth & Rovee-Collier, 1999). This reactivation effect does

not support a storage decrement hypothesis; reminder treatments likely act to increase accessibility of the stored memory for retrieval (Rovee-Collier, 1999).

The field of developmental psychobiology is largely in agreement that retrieval failures contribute significantly to infant memory impairments and recent work in rodents has also yielded converging evidence for retrieval failures in infancy (Josselyn & Frankland, 2012; S. Li et al., 2014; Madsen & Kim, 2016; Rovee-Collier & Giles, 2010). Infant mice (PD 17) trained on the Morris water maze will preferentially swim in the area previously occupied by a hidden platform when tested 1 day after training but not 30 day after training (Guskjolen et al., 2016). However, when given a reminder (short placement on the target platform) 1 hr prior to testing (after a 30 day retention) memory for the platform location is reactivated. Similarly, memory for an inhibitory avoidance task acquired by infant rats is forgotten within a day but can be reinstated following re-exposure to the training context on one day and foot-shock on another day (Travaglia, Bisaz, Sweet, Blitzer, & Alberini, 2016b). Interestingly, the reactivation effect observed by Guskjolen et al. (2016) was transient, with no beneficial effects of the reminder observed if given 24 hr (rather than 1 hr) prior to the probe trial. In contrast, Travaglia et al. (2016b) did show a reactivation effect with the reminder shock 24 hr prior to testing and even when the training context re-exposure and reminder foot-shock were separated by up to 7 days. This suggests time sensitivity of the reminder treatment that is specific to the type of memory (i.e. spatial vs contextual). The time sensitivity of reminder effects has been observed in humans (Hildreth & Rovee-Collier, 1999) and is possibly the result of an interaction between the age of the subject and neurological processing speed for the reminder cue or the to-

be remembered item. Whether changes in processing speed across ontogeny contributes to infantile memory deficits is an interesting area to explore.

3.3 Neurobiological Contributions to Infant Memory Development

Developing in parallel with psychological theories of infantile amnesia are theories regarding the neurobiological basis of the infant memory development. One idea is that the neurologically "immature brain" may be capable of initially forming a memory, however components of a given memory circuit (e.g. the hippocampus, prefrontal cortex or amygdala) are not sufficiently developed to support long-term retrieval of memory traces (Callaghan et al., 2014; Josselyn & Frankland, 2012; Madsen & Kim, 2016). This is illustrated by the idea that immaturity of mPFC, the region responsible for remote memory retrieval (Frankland & Bontempi, 2005; Wiltgen & Tanaka, 2013), is a mechanism of infantile amnesia (see below for detailed discussion). An alternate hypothesis is that brain maturation *after* the period when memories are formed somehow interferes with storage or retrieval of long-term memory, for example the hippocampal neurogenic hypothesis. This hypothesis posits that replacement of preexisting neuronal circuits, due to high rates of hippocampal neurogenesis, causes a failure of long-term declarative-like memory for events that occur when neurogenesis levels are high (Frankland, Kohler, & Josselyn, 2013; Josselyn & Frankland, 2012). In support, there is an inverse relationship between hippocampal neurogenesis and long-term retention of hippocampus-dependent learning that occurs prior to neurogenesis (Akers et al., 2014; Altman & Das, 1965, 1966). Differences in infantile amnesia between precocial species (e.g., guinea pigs) and altricial species (e.g., mice and rats) also illustrate this inverse relationship (Akers et al., 2014; Campbell et al., 1974). More direct tests of the neurogenic hypothesis

have manipulated hippocampal neurogenesis in infant and adult rats to determine a functional contribution of hippocampal neurogenesis to memory deficits. Increasing neurogenesis in adult mice following contextual fear learning significantly impairs retrieval performance at test while reducing neurogenesis in infants rescues their memory deficits (Akers et al., 2014). These results support the neurogenic hypothesis as one possible neurological mechanism for infantile amnesia.

In addition to the hippocampus, neuronal maturation in other brain regions likely contributes to the ontogeny of long-term memory for forms of learning that depend on those regions. The amygdala shows significant neurophysiological maturation during the first few postnatal weeks (M. Davis, Rainnie, & Cassell, 1994; D. E. Ehrlich, Ryan, Hazra, Guo, & Rainnie, 2013; D. E. Ehrlich, Ryan, & Rainnie, 2012; I. Ehrlich et al., 2009). Gogolla, Caroni, Luthi, and Herry (2009) showed that a specific type of extracellular matrix called perineuronal nets (PNNs) may contribute to long-term memory of amygdala-dependent learning. Amygdala levels of PNNs increase substantially between PD 16 and 21 and continue to increase until PD 28 when they reach near adult levels. In addition, degrading PNNs in adult mice caused memory "erasure," similar to that observed in young mice, during fear retrieval tests (Gogolla et al., 2009). Taken together, these results suggest that developmental changes in amygdala structure and neurophysiology likely support stable, long-term memory of learning that depends on the amygdala in young rodents.

In addition to the hippocampus and the amygdala, the prefrontal cortex also undergoes substantial postnatal maturation. The individual layers of the prefrontal cortex can be observed by day 10 however cellular differentiation within the layers produces significant cytoarchitectural changes through PD 18 (Van Eden & Uylings,

1985). The time between PD 15 and PD 20 seems to be an early transitional period where the cellular properties of medial prefrontal cortex begin to show rapid maturation in synaptic responses to stimulation, short-term plasticity, and pyramidal cell morphology (Zhang, 2004). These structural studies reveal that although prefrontal volume continues to develop well into young adulthood (Van Eden & Uylings, 1985), stable intrinsic properties emerge around weaning and this likely contributes to the emergence of prefrontal-dependent behaviors including retrieval of remote memories. Li, Kim and Richardson (2012) used a cued-fear conditioning procedure to explore the possible involvement of the PL region of the mPFC in fear conditioning between animals trained at PD 16 and PD 23. In adult animals, the PL works collaboratively with the IL and the amygdala to control fear memory expression following conditioning (for review see Sotres-Bayon & Quirk, 2010). Similarly, inactivation of the PL in juvenile (PD 23) rats reduced fear expression to the conditioned cue during testing and PL protein expression following retrieval was correlated with performance of the conditioning group (i.e. paired vs. unpaired training; S. Li, Kim, & Richardson, 2012a). Conversely, rats trained at PD 16 showed considerable levels of freezing to the paired CS-US presentations but their rates of protein expression in PL did not differ from the controls. Furthermore, inactivation of the PL did not decrease conditioned freezing to the stimulus suggesting that the prefrontal cortex is not necessary for fear memory expression in infant rats. Additionally, these results suggest there are fundamentally different memory retrieval systems that possibly do and do not rely on the maturation of the prefrontal cortex. Callaghan et al. (2014) proposed that in adults, the prefrontal cortex interacts with the BLA and CEn of the amygdala to support fear memory expression however that

circuit is likely much simpler in preweanling rats when it is comprised of the amygdala alone.

To summarize this section as a whole, the transition from high levels of granule cell neurogenesis in the hippocampal formation during development to lower levels in adolescence and adulthood is associated with increased long-term memory capacity for hippocampus-dependent learning (Akers et al., 2014; Frankland et al., 2013; Josselyn & Frankland, 2012; Madsen & Kim, 2016). Additionally, long-term memory for fear conditioning may rely on the development of memory circuits within the amygdala and/or it's interactions with the developing prefrontal cortex or hippocampus (Tallot, Doyere, & Sullivan, 2016).

3.4 The Debate over Infant Context Learning: An Unresolved Issue.

Contextual fear conditioning has been widely used to study the neurobiology of learning and memory in both adult and developing animals. Juvenile (PD 23) but not infant (PD 18) rats readily show test freezing 24 hr after context-foot-shock training (Figure 3.3a; Rudy, 1993; Rudy & Morledge, 1994; Schiffino et al., 2011). The earliest accounts of this developmental trend posited a failure to acquire a context representation at PD 18 because the hippocampus is not sufficiently mature at this age, relative to PD 23 (Rudy, 1993).

This view that infant rats could not acquire context representations was contradicted when subsequent studies showed that infant rats could show strong contextual conditioning when the retention interval between context-shock training and testing was reduced to zero by measuring post-shock freezing following the conditioning trial (Figure 3.3b). With this measure, Rudy and Morledge (1994) argued that age differences in sCFC reflect differences in long-term (24 hr) retention of

context representations rather than age differences in encoding contextual cues or forming a context-shock association. In adults, short-term contextual conditioning (post-shock freezing) can be pharmacologically dissociated from long-term memory when hippocampal injections of NMDA-receptor antagonists precede conditioning (J. J. Kim, Fanselow, Decola, & Landeirafernandez, 1992). This suggests the existence of two temporally dissociable context-learning systems. The infant sCFC expression patterns essentially mimic what is observed in this adult study and support an account of impaired sCFC during development that reflects poor long-term (24 hr) memory.

Attempts to further characterize the nature of infant context learning and memory led experimenters to investigate the ability of infant rats to build and maintain a conjunctive representation of their training environment. Without time to construct a conjunctive context representation, rats show impaired fear conditioning to an immediate foot-shock. This immediate shock deficit (ISD) is alleviated with context preexposure 24 hr prior to immediate foot-shock (the CPFE). Rudy and Morledge (1994) suggested that if young rats were able to create a conjunctive representation then they should benefit from context preexposure, similar to adults. Indeed, juvenile rats (PD 23) did show significant fear conditioning using the CPFE and our subsequent studies showed that the CPFE fully develops between PD 21 and PD 24 without further development during adolescence or adulthood. In contrast, rats aged PD 17 or 19 fail to show the CPFE (Figure 3.4; Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015; Rudy & Morledge, 1994; Schiffino et al., 2011). Although infant rats are able learn contextual fear, these results suggests that infant rats do not construct a conjunctive representation of the context that is retrievable 24 hr later for association with the immediate shock on the training day.

This claim that pre-weanling rats (PD 16-20) fail to learn or remember contextual cues has been challenged in a recent review by Revillo et al. (2015). They report mixed results from 20 studies (including those mentioned here) that have appeared since 1990 (Table 1 in their article). Long-term (24 hr) retention was found in 8 of the 20 studies. Positive findings were associated with more salient context cues or repeated trials (e.g. multiple foot-shocks). For example, when solid black-walled training chambers defined the context (rather than clear walls surrounded by distal cues), Pugh and Rudy (1996) demonstrated similar freezing in PD 18 and PD 23 rats. However, many of the positive findings in Revillo's report may reflect brightness discriminations or odor-shock learning rather than context conditioning. Importantly, in Pugh and Rudy (1996), conditioning was evident when preexposure was to the full context or to its individual features. This suggests that the "contextual conditioning" observed in infant rats was likely based on non-conjunctive, elemental processing (Fanselow, 1990; Rudy, 2009) rather than hippocampus-dependent unitary conjunctive representations demonstrated to be utilized by PD 24 rats (Jablonski et al., 2012; Schiffino et al., 2011). In summary, it is clear that contextual associations are acquired in infants and older rats, however infant rats may not form the conjunctive context representations used for long-term retention.

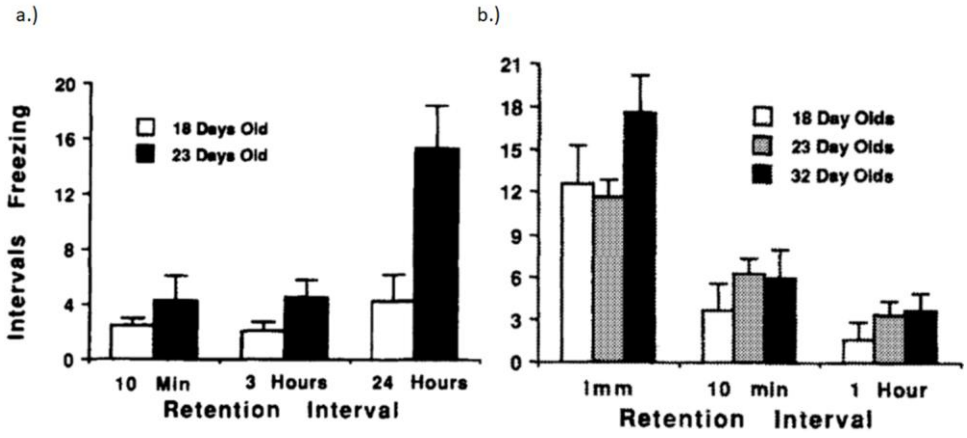

Figure 3.3 Mean intervals of freezing during test of contextual fear conditioning (sCFC) in developing rats as a function of retention interval between fear conditioning and test. Animals were given 2 min to freely explore the conditioning chamber before receiving a single foot-shock. a) Test for sCFC was assessed 10 min, 3 or 24 hr after conditioning. CFC was not evident in PD 18 rats, however fear was robust when examined 24 hr after training in PD 23 rats. b.) Test for sCFC was assessed immediately (Imm), 10 min or 1 hr after conditioning. When testing for fear happened immediately following conditioning PD 18 animals showed robust fear that was not different from that observed in PD 23 or PD 32 animals indicating comparable levels of fear acquisition. Images taken from Rudy and Morledge, 1994.

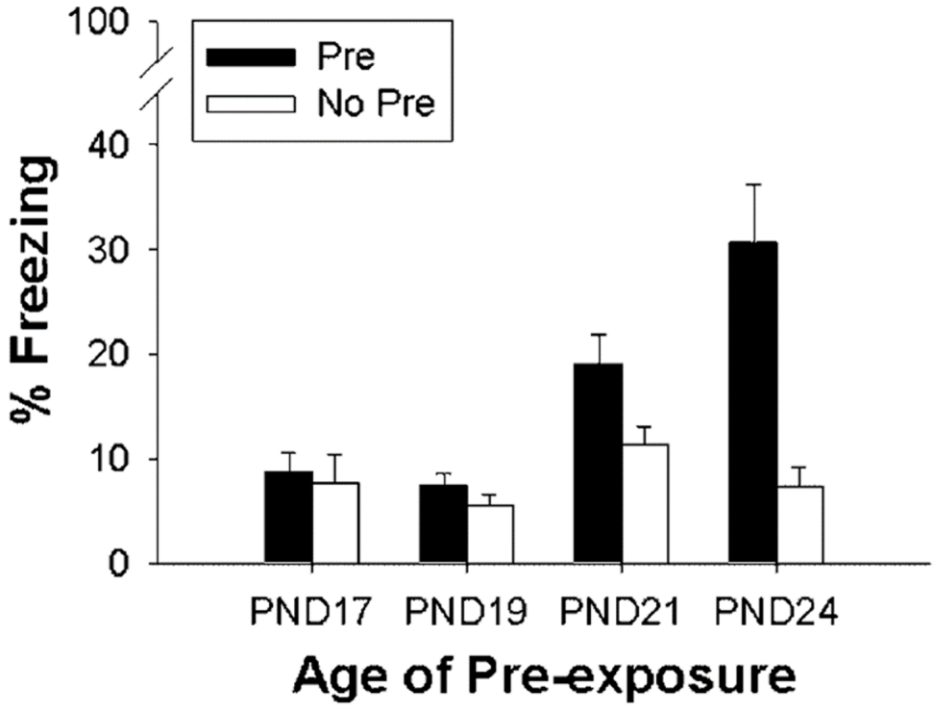

Figure 3.4 Mean (±SEM) percent freezing during test of the CPFE as a function of preexposure age (PD 17, 19, 21, and 24). Animals were preexposed to the training context (black bars) or an alternate context (white bars) to that used during context-shock training. The beneficial effects of context preexposure on fear conditioning are mild at PD 21 and strongest beginning at PD 24 however no conditioning was evident when preexposure happened on PD 17 or 19. Image taken from Jablonski et al., 2012.

3.5 Conclusions

The current chapter illustrates that a great deal is still unknown about the nature of infant learning and memory processes. Early results in humans and rodent models suggest that memory acquisition may be equivalent between infant and adult animals and that retrieval deficits underlie poor performance of infants on long-term memory tests. When specifically modeling hippocampus-dependent learning, studies using contextual fear conditioning suggests that infant rats can perceive and associate contextual cues with shock but they fail to build a conjunctive context representation that is necessary for performance during long-term memory tests. In an effort to neurobiologically characterize the mechanisms of learning and memory during development, recent reports have combined molecular measures with behavioral manipulations and assessed regional activity in response to learning as a function of age (Deal, Erickson, Shiers, & Burman, 2016; J. H. Kim, Li, Hamlin, McNally, & Richardson, 2012; Travaglia, Bisaz, Cruz, & Alberini, 2016a; Travaglia et al., 2016b). Accordingly, the current dissertation uses molecular assays during the CPFE to further characterize ontogenetic differences in contextual fear learning and memory.

Chapter 4

FETAL ALCOHOL SPECTRUM DISORDER

Prenatal alcohol exposure in humans causes a group of neurological and behavioral impairments known as Fetal Alcohol Spectrum Disorder (FASD). This umbrella term is used to describe a range of developmental effects that, at their most severe, is termed Fetal Alcohol Syndrome, a disorder characterized by facial dysmorphology, growth impairments and severe neurological and cognitive deficits (Del Campo & Jones, 2017; Senturias & Asamoah, 2014). Although educational campaigns which inform the public about the deleterious effects of prenatal alcohol exposure have striven to reduce the incidence of FASD, it is estimated that 2-5% of school aged children in the United States and Western European countries have some form of the disorder (*Fetal Alcohol Spectrum Disorders (FASDs): Data and Statistics*, 2017). Furthermore, it is estimated that the cost for Fetal Alcohol Syndrome alone is $4 billion annually in the United States making FASD a devastating disorder both personally and economically. Human and animal research into FASD has been invaluable in elucidating the neurobiological substrates of the disorders with key regions like the hippocampus, cerebellum, corpus callosum, and frontal cortex being particularly susceptible to the teratogenic effects of early alcohol exposure (Lebel et al., 2011). Environmental, biological and genetic factors interact to determine the diagnosis and severity of cognitive-behavioral outcomes (Guerri, Bazinet, & Riley, 2009; Lebel et al., 2011). Although cognitive-behavioral deficits observed in human FASD have been difficult to model in laboratory animals at times (Kelly, Goodlett, & Hannigan, 2009; Patten, Fontaine, & Christie, 2014), animal models have strengthened previous hypotheses regarding the mechanisms by which prenatal alcohol exposure

produces its detrimental effects and contributed new insights into possible treatments. Understanding the mechanisms of FASD is critical to helping reduce the negative outcomes associated with prenatal alcohol exposure and facilitating healthy cognitive-behavioral development post-parturition.

4.1 FASD in Developing Humans

Structural and functional assessment of neurological changes associated with prenatal alcohol exposure has revealed significant developmental brain alterations in children following *in utero* alcohol exposure. Overall, alcohol exposure causes a global reduction in total brain volume (Lebel et al., 2011). Individual evaluations of white and gray matter have revealed differential developmental trajectories between FASD and non-exposed control children. Cortical gray matter volumes show an inverted U-shaped pattern across development in non-exposed children while FASD children show only decreases in volume (Lebel et al., 2012). In contrast, growth rates in white matter and subcortical gray matter, which were still reduced overall, were not significantly different between groups (Gautam et al., 2015; Gautam, Nunez, Narr, Kan, & Sowell, 2014; Lebel et al., 2012). In addition, functional connectivity (e.g. processing efficiency) is more likely to be atypical in alcohol-exposed children when compared to age-matched controls (Wozniak et al., 2016). Preliminary attempts to phenotype the cortical profiles of FASD children using computational neuroimaging have revealed consistent alterations in cortical morphology (e.g. reduced cortical surface area) although some discrepant findings encourage further exploration and replication of these potential cortical markers of FASD (for review see De Guio, Meintjes, Mangin, & Germanaud, 2016).

4.2 FASD in Developing Animal Models

Animal models have been used to explore the neurological basis for the teratogenic effects of alcohol on the developing brain. Alcohol exposure during embryonic day (ED) 7 or 8 in mice (Lipinski et al., 2012; Sulik, 2005) or ED 19 or 20 in macaques (Astley, Magnuson, Omnell, & Clarren, 1999) leads to FAS-associated facial dysmorphology while exposure from gestational day (GD) 12 to 20 in rats disrupts neuroepithelial cell proliferation and migration (Guerri et al., 2009). Although craniofacial dysmorphology is associated with early alcohol exposure, reports suggests that severe cognitive impairments result from alcohol exposure during the period of rapid synaptogenesis and neurogenesis known as the "brain growth spurt" (Dobbing & Sands, 1979; Guerri et al., 2009; Klintsova, Hamilton, & Boschen, 2012; Patten et al., 2014). Due to the altricial state of rodents at birth, the period of time equivalent to the third trimester in humans, in term of intense neurogenesis, is roughly equivalent to the first 10 postnatal days (Dobbing & Sands, 1979). As detailed in Sections 4.3-4.7 (below), alcohol exposure during this time disrupts development of the hippocampus (Gil-Mohapel, Boehme, Kainer, & Christie, 2010; G. F. Hamilton et al., 2011; Livy, Miller, Maier, & West, 2003; Miller, 1995; Murawski, Klintsova, & Stanton, 2012; Tran & Kelly, 2003) and prefrontal cortex (G. F. Hamilton et al., 2010c; Lawrence, Otero, & Kelly, 2012; Whitcher & Klintsova, 2008), two regions involved in forms of cognition that are impaired by neonatal alcohol exposure (Guerri et al., 2009; Mattson, Crocker, & Nguyen, 2011; Mattson & Riley, 2011).

4.3 FASD and the Hippocampus

Hippocampus structural and functional abnormalities have been widely reported in humans following prenatal alcohol exposure and hippocampal dysfunction

has been proposed as a mechanism for cognitive impairments in FASD children (Guerri et al., 2009; Mattson et al., 2011; Norman et al., 2009). Derek A. Hamilton, Kodituwakku, Sutherland, and Savage (2003) used a virtual place-learning task to examine hippocampus-dependent spatial abilities in children with FASD. Similar to effects observed in hippocampal-lesioned rats (Morris, Garrud, Rawlins, & O'Keefe, 1982) and rats neonatally exposed to alcohol (Johnson & Goodlett, 2002; Kelly, Goodlett, Hulsether, & West, 1988), children with FASD showed significantly longer latencies to find the platform when it was hidden relative to control children although latencies were identical when the platform was visible. Similarly, using a memory for objects task, Uecker and Nadel (1996) examined immediate and long-term spatial memory for an array of 16 objects. They found that FASD children performed similarly to control children at the immediate test of object recall, however, during a 24 hr retention test, FASD children performed significantly worse. Preserved performance during control tasks suggests a specific effect of alcohol-induced brain damage on spatial learning processes that are not secondary to sensory effects such as visuospatial acuity. Corroborating evidence from imaging studies that report impaired spatial memory with reduced hippocampal volume in children (Willoughby, Sheard, Nash, & Rovet, 2008) converge with the previous results to suggest that hippocampus-dependent spatial memory is one aspect of cognitive impairment observed in FASD children.

4.4 FASD and the Hippocampus Morphology

Severity of teratogenic effects following prenatal and neonatal alcohol exposure can vary due to many factors. Different developmental periods (“windows”) of exposure, doses, or pattern of exposure (e.g., binge-like peaks vs. steady, low

levels) can all dramatically alter the magnitude of subsequent neurological damage and cognitive impairment (Maier & West, 2001; May et al., 2013; May & Gossage, 2011). Rodent models that systematically control and manipulate these factors have revealed a particular susceptibility of hippocampal development and physiology when alcohol exposure occurs during periods equivalent to the human 3rd-trimester (Gil-Mohapel et al., 2011; Livy et al., 2003; Tran & Kelly, 2003). Following alcohol exposure across dosing windows equivalent to all three human trimesters (ED 1 to PD 9) pups sacrificed on PD 10 showed reductions in hippocampal cell number in CA1, CA3 and the dentate gyrus (Bonthius & West, 1990; Livy et al., 2003). Interestingly, when dosing windows were reduced to simulate dosing over single or double trimester windows, the third trimester equivalent (i.e. PD 4-9) was the only dosing window to produce significant hippocampal damage despite similar blood alcohol levels across all exposure windows. Additionally, the type of damage observed in the hippocampal subregions was not uniform; all regions showed reduced cell number by approximately 50%, however, only CA1 showed a significant reduction in volume (Livy et al., 2003). Because third trimester dosing caused similar hippocampal damage as dosing across all three trimesters, rodent-model studies attempting to capture human hippocampal cognitive deficits have largely used this window (e.g., PD 4-9 in rodents).

In addition to these hippocampal effects observed early in development, structural changes observed after perinatal dosing persist throughout development into adulthood. Reductions in CA1 cell number of nearly 30% were evident in adolescent rats following third trimester alcohol exposure (Figure 4.1b; Murawski et al., 2012). Furthermore, in the adult hippocampus, lasting effects of third trimester alcohol

exposure were evident in CA1 with cell numbers reduced by nearly 17%, although reductions in CA3 and the dentate gyrus were not observed (Tran & Kelly, 2003). Dentate granule cells that undergo neurogenesis in adolescence show reduced survival (G. F. Hamilton et al., 2011) while pyramidal cells show reduced apical and basilar spine number and density with variations in spine type (e.g. thin vs stubby) that is evident in adolescence but also recovers by adulthood (Davies & Smith, 1981; Ferrer, Galofre, Lopeztejero, & Llobera, 1988; Tarelo-Acuna, Olvera-Cortes, & Gonzalez-Burgos, 2000). Although the hippocampus is generally dysregulated following alcohol exposure, these results confirm that CA1 is not only more sensitive to the initial effects of alcohol exposure, which lasts throughout development, but that CA3 and the dentate gyrus may be able to regain normal neuroanatomical features over the course of development.

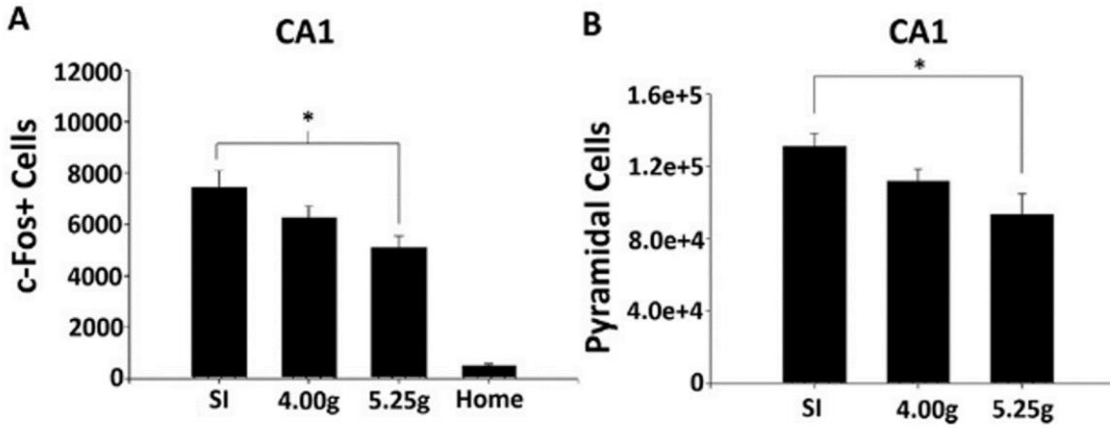

Figure 4.1 Evidence of hippocampal damage following PD 4-9 alcohol exposure. Mean (±SEM) estimates of CA1 *c-fos* positive (+) cells 2 h following context preexposure in animals neonatally exposed to 0.00g (SI), 4.00 g, and 5.25 g/kg/day alcohol. (B) Mean (±SEM) CA1 pyramidal cells in rats from Groups SI, 4.00 g, and 5.25 g. Asterisk"*" indicates significant group differences. CA1 cell number and *c-fos* positive cells were reduced in animals neonatally exposed to alcohol. Figure taken from Murawski, Klintsova and Stanton, 2012.

4.5 FASD and the Prefrontal Cortex

In addition to impairments in hippocampus-dependent learning and memory tasks, children and adults with FASD show significant impairment in cognitive processes thought to rely on the prefrontal cortex, such as working memory and executive function (Mattson et al., 2011; Mattson & Riley, 2011; Rasmussen, 2005). Using a battery of neuropsychological tasks to identify which cognitive domains are most affected by prenatal alcohol, Burden, Jacobson, Sokol, and Jacobson (2005) reported that aspects of attention and working memory, which function to link current information with previously encoded information, were most affected by prenatal alcohol exposure in humans. In a test designed specifically to evaluate spatial working memory called the *n*-Back task, Malisza et al. (2005) required FASD subjects and controls to identify the spatial location of a colored circle by pressing a button and manipulated whether there was simply the circle presented before responding, a blank card, or a series of one or two other stimuli (simple, blank, one-back and two-back tasks, respectively). Overall the FASD subjects performed worse than the controls although their performance was only statistically worse when comparing the one-back task to the simple and blank task (FASD responding was so poor during the two-back task that their results were dropped from data analyses). The effects were evident in both children and adults which suggests that spatial working memory deficits persist throughout the lifetime of FASD subjects.

Some disruptions in working memory have been correlated with dysfunction in specific prefrontal circuits. During a test of response inhibition, children between the ages of 8-18 years old with FASD diagnoses showed abnormal frontal-striatal activity such that during responding their frontal lobe activity increased while their caudate activity decreased (Fryer et al., 2007). In addition, Malisza et al. (2005), using the *n*-

Back task, also found increased frontal lobe activity both in children and adults with FASD relative to controls, especially in prefrontal regions. Furthermore, children (relative to adults) showed decreased frontal cortex functional activity with increasing task difficulty suggesting a potentially greater level of dysfunction during this task in children relative to adults. In summary, the high levels of impairment in FASD subjects during prefrontal-related cognitive tasks, imaging studies showing specific PFC malformation, and functional studies illustrating frontal lobe dysfunction during attention and working memory tasks, suggests that PFC damage contributes to attention and working memory deficits seen in FASD patients.

4.6 FASD and Prefrontal Morphology

Alterations in prefrontal function following prenatal alcohol exposure is likely the product of altered prefrontal structure and physiology (Lebel et al., 2011; Norman et al., 2009; Sowell et al., 2002; Zhou et al., 2011). During human fetal development, heavy alcohol consumption is associated with reduced fetal frontal cortex size early in gestation (Wass, Persutte, & Hobbins, 2001) that persists into the third trimester (Kfir et al., 2009). Disruptions in cortical developmental trajectories persist postnatally with structural abnormalities observed well into young adulthood (Lebel et al., 2012; Sowell et al., 2002; Zhou et al., 2011).

Using rodent models, abnormalities in prefrontal development that emphasize the effect of alcohol on specific aspects of cellular and synaptic morphology have also been reported. Following alcohol exposure across all trimesters, adult pyramidal cells in the mPFC show reductions in both apical and basilar spine density and a reduced number of dendritic branches (Lawrence et al., 2012). When examining cellular structure during adolescence, there is reduced apical spine density in alcohol-exposed

animals but no change in morphology or complexity (e.g. thin vs stubby morphology; Whitcher & Klintsova, 2008). For basilar dendrites however, complexity is changed with both reduced dendrite length and tree intersections proximal to the soma. Spine density overall is not changed but the morphological profile of spines does shift in alcohol-exposed animals to a high mature/low immature dendritic spine profile (G. F. Hamilton et al., 2010c). The authors postulate that differences in thalamic innervation may contribute to altered prefrontal morphology. The mPFC has distinct thalamic innervation across its subregions and layers (Ferguson & Gao, 2014; Heidbreder & Groenewegen, 2003; Varela, Kumar, Yang, & Wilson, 2014) that are altered following prenatal alcohol exposure (Granato, Santarelli, Sbriccoli, & Minciacchi, 1995). Synapse-level dysregulation of afferent projections to the prefrontal cortex may produce morphological changes in the cells onto which these afferents project.

There is evidence to suggest that the morphological changes observed after alcohol exposure may result in alterations of plasticity-related functions both in terms of basal gene expression and in response to experience. Immediate early genes (IEGs) like *Egr-1, c-fos,* and *Arc* are molecular components of learning and long-term memory processes that are upregulated following experience in regions implicated in alcohol-related cognitive impairments (Alberini, 2009; Knapska & Kaczmarek, 2004; Okuno, 2011). Following maternal subcutaneous alcohol administration, alcohol-exposed infant (3-weeks-old) and adolescent (5-weeks-old) rats have reduced numbers of *c-fos* positive cells in CA1, CA2-3 and the dentate gyrus (Jang et al., 2005). Frontal levels of *Arc* and *c-fos*, which increase in response to social experience in control rats, were not increased in alcohol-exposed rats (D. A. Hamilton et al., 2010a; D. A. Hamilton et al., 2010b). Importantly, during a spatial learning task when

alcohol-exposed rats show impairments in memory, both prefrontal (Nagahara & Handa, 1995) and hippocampal (Clements, Girard, Ellard, & Wainwright, 2005) IEG levels are reduced in their expression relative to non-exposed control animals. These results support a role of reduced plasticity-related molecular function in the hippocampus and prefrontal cortex of alcohol-exposed animals that likely contributes to cognitive impairments thought to rely on these regions.

4.7 Disruption of the CPFE Following Alcohol Exposure

The impairments observed in the hippocampus and prefrontal cortex inform our understanding of how alcohol exposure results in impairment of complex cognitive processes. As reviewed previously (Chapter 2), contextual fear conditioning requires both the hippocampus and the prefrontal cortex (Gilmartin, Balderston, & Helmstetter, 2014; Giustino & Maren, 2015; Heroux et al., 2017; Maren et al., 2013; Zelikowsky et al., 2013; Zelikowsky et al., 2014) and the CPFE specifically is disrupted following alcohol exposure (Figure 4.2; Dokovna et al., 2013; G. F. Hamilton et al., 2011; Jablonski & Stanton, 2014; Murawski et al., 2012; Murawski & Stanton, 2010, 2011). Third-trimester equivalent binge-doses of alcohol (5.25g/kg/day from PD 4-9) abolishes the CPFE (Murawski & Stanton, 2010) and this disruption is critically dependent on the dosing window; exposure from PD 4-9 or PD 7-9 impairs the CPFE but exposure from PD 4-6 does not (Murawski & Stanton, 2011). Behavioral dose-response curves following various alcohol doses from PD 4-9 or PD 7-9 revealed that the CPFE deficit increases in a linear manner as a function of alcohol dose when dosing occurs from PD 4-9 (Figure 4.2b; Murawski & Stanton, 2011). However, given the same alcohol doses during PD 7-9, alcohol only produces a CPFE deficit at the highest dose (5.25g/kg/day; Figure 4.2a; (Jablonski & Stanton, 2014).

Additionally, 24 hr retention was impaired in alcohol-exposed rats, but immediate post-shock freezing revealed preserved contextual fear conditioning suggesting that context-shock association consolidation or testing-day retrieval is likely the psychological basis for disrupted CPFE performance in animals exposed to alcohol on PD 7-9 (Jablonski & Stanton, 2014). These results illustrate the sensitivity of the CPFE to neonatal alcohol exposure and suggests that the PD 4-9 and PD 7-9 dosing windows may target different neural substrates or systems. Additionally, hippocampal IEG expression (as measured by *c-fo*s positive cell count) was altered in PD 4-9 alcohol-exposed animals relative to controls following context preexposure during the CPFE (Figure 4.1a; Murawski et al., 2012). This suggests that neonatal alcohol exposure impairs the CPFE by disrupting normal hippocampal function. As both the hippocampus and the prefrontal cortex are necessary for CPFE performance (Heroux et al., 2017; Matus-Amat et al., 2004; Matus-Amat et al., 2007; Robinson-Drummer et al., 2016) it is likely that analyses of prefrontal IEG activity during the CPFE will reveal similar impairments.

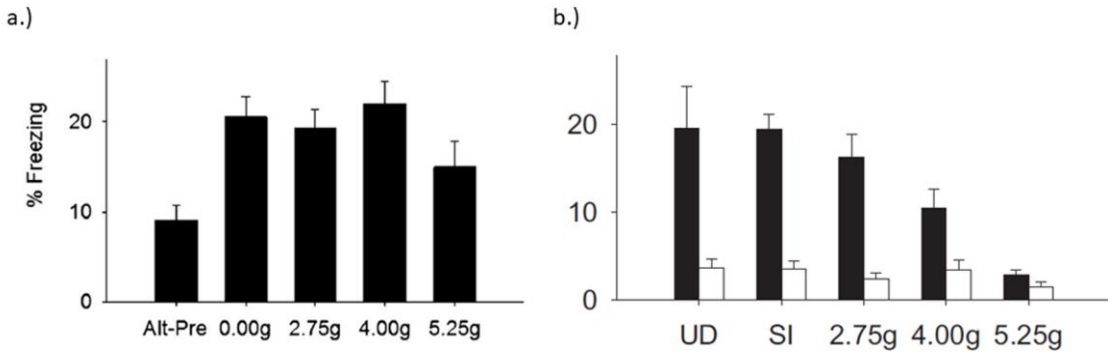

Figure 4.2 Mean (±SEM) percent freezing during testing for the CPFE following 0.00 (SI), 2.75, 4.00, and 5.25 g/kg/day alcohol from a.) PD 7-9 or b.) PD 4-9. a.) PD 7-9 dosing only reduced freezing at the highest dose (5.25g) relative to SI controls (0.00g). b.) Following PD 4-9 exposure, test freezing was graded and decreased as a linear function of alcohol dose. Group Alt-Pre freezing (far left bar in graph a. and white bars in graph b.) was low regardless of dosing condition). Figure a.) taken from Jablonski and Stanton 2014. Figure b.) taken from Murawski and Stanton, 2011.

4.8 Conclusions

Human studies and rodent models of fetal alcohol spectrum disorder reveal a significant disruption of hippocampal function and abnormal structure across the lifespan of alcohol-exposed subjects. These alterations are associated with behavioral and cognitive impairments in tasks critically dependent on hippocampal function for successful performance. Additionally, the prefrontal cortex, which is also damaged following alcohol exposure, is similarly disrupted both in terms of cellular activity and proper function during learning and behavior. Evidence suggests that the structural and functional disruptions in these regions that are associated with learning and memory impairments may be mediated by dysfunction in molecular activity that serves to facilitate long-term learning and memory processes. Specifically, neonatal alcohol exposure impairs consolidation or retrieval of the context-shock association in rats during the CPFE. The current dissertations seeks to extend this literature by exploring the response of an understudied IEG in FASD research (*Egr-1*) during the CPFE in order to propose a mechanism by which memory during this task is impaired following neonatal alcohol exposure.

Chapter 5

PRELIMINARY STUDIES

The preceding chapters reviewed the neurobiology and ontogeny of contextual fear learning and memory as well as the neural and behavioral consequences of developmental alcohol exposure on hippocampal and prefrontal learning processes. The current chapter will present previously published studies that were the foundation for the experiments chosen for this dissertation. These foundational studies highlight 1) the psychological basis for developmental differences in the CPFE 2) the molecular processes involved in contextual fear conditioning 3) and the involvement of muscarinic-type acetylcholine receptors in each phase of the CPFE.

5.1 Ontogeny and Behavioral Determinants of the CPFE

The CPFE requires that a previously acquired context representation be retrieved through pattern completion and associated with an immediate foot-shock (Chapter 1; Rudy, 2009). In Schiffino et al. (2011), rats were preexposed, 24 hr prior to foot-shock training, to either the training context (Context A) or an alternate context (Context B). On the training day, foot-shock training occurred following a 120 s placement-to-shock interval (PSI; sCFC groups) or a 0 s PSI (CPFE groups) in Context A. Twenty-four hours after training, rats were returned to Context A and tested for contextual fear memory. Whereas animals preexposed at PD 24 and PD 31 showed significant contextual fear using both procedures, preweanling (PD 17) rats showed no evidence of conditioning regardless of preexposure or PSI condition. The results suggest that PD 17 rats either are unable to acquire a contextual representation, associate a retrieved representation with foot-shock or retrieve a previously formed contextual fear memory.

As noted in Chapters 3, infant rats are able to acquire and momentarily associate a context representation with foot-shock but long-term retention (24 hr) of contextual fear does not emerge until after weaning (Jablonski et al., 2012; Rudy & Morledge, 1994; Schiffino et al., 2011). Based on the findings from Rudy and Morledge (1994), it is possible that the CPFE failure observed in PD 17-19 rats (Jablonski et al., 2012; Schiffino et al., 2011) with 24 hr between foot-shock training and test may be a consequence of infant rats being unable to consolidate the context-shock association into a retrievable memory trace. This hypothesis was refuted by showing that even when testing occurred immediately following foot-shock, infant rats showed no benefit of context preexposure and failed to show conditioning (Jablonski et al., 2012). In this case, infant rats, that are capable of context learning and acquiring a context-shock association (Rudy & Morledge, 1994), either cannot consolidate and retrieve the context representation acquired during preexposure or cannot associate a retrieved memory with foot-shock.

Jablonski et al. (2012) suggested that PD 17 rats are incapable of consolidating the contextual memories they acquire on the preexposure day for long-term storage and retrieval which is why they fail to learn the CPFE but show robust fear conditioning when exposure, training and testing occur in the same training episode (Rudy & Morledge, 1994). In mice, memory for sCFC can be retained for at least 28 days in both adult and adolescent animals (Akers et al., 2012). However infant mice, which show normal retention 24 hr after training, show no evidence of fear memory at 7 days illustrating a deficit in fear memory retention that diminishes between infancy and adolescence. The previous study suggested that contextual fear memories are not maintained when learning occurs in infant animals however their results do not specify

whether the memory deficit was for the context-shock association or the context representation alone. Rudy and Wright-Hardesty (2005) showed that adult animals can retain an incidentally acquired context memory for up to 28 days. In order to explore the ontogeny of context-only memory retention we first examined the retention abilities of juvenile (PD 24) and adolescent (PD 31) rats using several intervals between preexposure to the training context and immediate foot-shock training (Robinson-Drummer & Stanton, 2015). Then we tested adult (PD 52) and infant (PD 17) context memory across similar intervals. We hypothesized that retention abilities would strengthen with age at exposure in support of the consolidation failure proposed by Jablonski et al. (2012).

The CPFE procedure took place in three phases: preexposure, training and testing with the preexposure-to-training interval varying across groups while the training and testing sessions always occurred 24 hr apart. On the preexposure day, pups were preexposed using a multiple preexposure procedure (see Chapter 6: Behavioral Procedures; Dokovna et al., 2013; Matus-Amat et al., 2004) to either Context A (Group Pre) or an alternate Context B (Group Alt-Pre). We utilized four retention intervals between preexposure and training: 1 day (replicating the conventional CPFE), 8 days, 15 days and 22 days. After the designated retention interval, all pups (Pre and Alt-Pre) were trained in Context A and tested in Context A 24 hr later.

In our first experiment, we examined how long juvenile (PD 24) and adolescent (PD 31) rats could retain a context memory beyond the comparable levels of 24 hr retention observed at these ages (Schiffino et al., 2011). As shown in Figure 5.1, both PD 24 and PD 31 rats in Group Pre retained the context memory for 1 day

(replicating Jablonski et al., 2012; Schiffino et al., 2011), 8 days and 15 days and displayed robust freezing (relative to Alt-pre controls) to the training context during testing. However, neither age showed a significant CPFE after the 22 day retention interval, revealing a failure to retain or retrieve the context representation after 22 days. In contrast, adult rats showed robust freezing after a 22 day retention interval between context preexposure and foot-shock training (Figure 5.2)

Across the developmental stages examined in this study, it appears that the ability to retain a context memory does not appear until after PD 17 (which showed no retention at any interval) and is not fully developed until young adulthood when rats are able to maintain the context memory for three weeks or more (see PD 52 in Figure 5.2; Rudy & Wright-Hardesty, 2005). The results of these experiments support a deficit either in consolidation processes on the preexposure day that are necessary for long-term storage or retrieval failure on training day. This study revealed a novel feature of context learning and memory over ontogeny and was foundational to the experimental data presented in the following chapters which explored ontogenetic changes in molecular activity thought to underlie consolidation processes on the preexposure and training day (see Alberini, 2009).

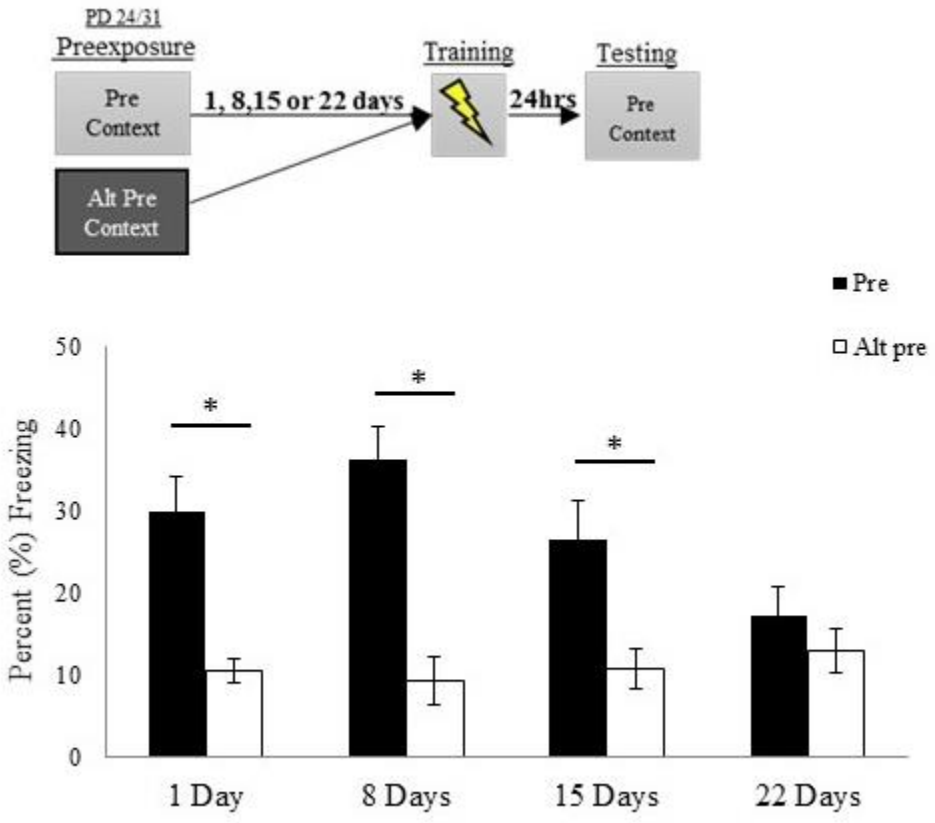

Figure 5.1 (Top) Schematic of CPFE timeline with preexposure occurring on PD 24 or 31. (Bottom.) Mean (±SEM) context freezing 24 hr after immediate-shock training in animals preexposed to the training context (Pre) or alternate context (Alt-Pre) on PD 24 or 31 (collapsed across age) either 1, 8 , 15 or 22 days before training. Asterisks "*" represent results of Newman-Keuls post-hoc analyses of retention x preexposure condition ANOVA with $p < .05$.

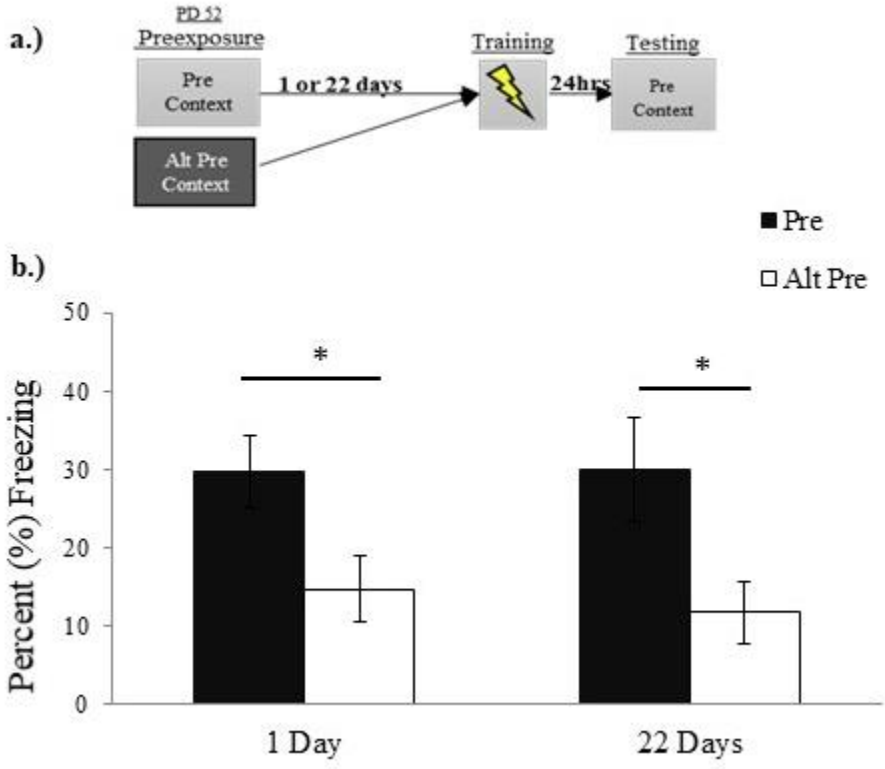

Figure 5.2 Panel a.) Schematic of CPFE timeline with preexposure occurring on PD 52. Panel b.) Mean (±SEM) context freezing 24 hr after immediate-shock training in animals preexposed to the training (Pre) or alternate context (Alt-Pre) on PND 52 either 1 or 22 days before training in the context. Asterisks "*" represent results of Newman-Keuls post-hoc analyses of retention x preexposure condition ANOVA with $p < .05$.

5.2 Molecular Processes Involved in Contextual Fear Conditioning

As reviewed in Chapter 2, the hippocampus and medial prefrontal cortex (mPFC) cooperatively support fear conditioning to contextual stimuli. Importantly, both regions participate in the acquisition and/or consolidation of the context representation and context-shock association as pharmacological antagonism of receptors or inhibition of local plasticity in these regions during preexposure or training disrupts conditioning (Chang & Liang, 2012; Heroux et al., 2017; Matus-Amat et al., 2004; Matus-Amat et al., 2007; Robinson-Drummer et al., 2016; Schiffino et al., 2011). In order to understand the molecular cascades that underlie these processes a large body of work has emerged documenting molecular changes in these regions during contextual fear conditioning (Alberini, 2009, 2011; Veyrac, Besnard, Caboche, Davis, & Laroche, 2014).

Early growth response-1 gene (*Egr-1*) is an inducible transcription factor involved in the long-term molecular changes associated with learning (Alberini, 2009; Donley & Rosen, 2017; Jones et al., 2001; Maddox, Monsey, & Schafe, 2011; Veyrac et al., 2014). In adults, reduction of amygdalar *Egr-1* levels during sCFC or hippocampal *Egr-1* levels during the CPFE significantly impairs fear conditioning (J. L. Lee, 2010; Malkani & Rosen, 2001; Malkani et al., 2004). Utilizing the CPFE, our lab sought to examine the molecular profile of *Egr-1* expression following context preexposure and context-shock association in order to characterize molecular changes associated with contextual fear conditioning in adolescent and adult rats (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014).

Beginning on PD 31, rats were preexposed to training context A (Group Pre) or to an alternate chamber context B (Group Alt-pre) using a single 5 min exposure (Asok et al., 2013). Thirty-minutes after the end of context preexposure both groups,

plus a non-preexposed homecage (HC) “baseline” group, were sacrificed and their brains removed for *in situ* hybridization (see Chapter 6: *In situ* hybridization). The medial prefrontal cortex [mPFC; prelimbic cortex (PL), infralimbic cortex (IL), anterior cingulate cortex (AC), orbitofrontal cortex (OFC)], dorsal hippocampus (dHPC) and lateral amygdala (LA) were assessed for *Egr-1* mRNA expression. Asok et al (2013) found that on the preexposure day, *Egr-1* expression significantly increased in all prefrontal subregions, the LA and the CA1 relative to the HC controls demonstrating neuronal-plasticity-related activity in all regions in response to novel context exposure (Figure 5.3).

Asok et al (2013) next asked if *Egr-1* expression would vary associatively (between Alt-Pre and Pre groups) on the training day. Rats were sacrificed 30 (±3) min after the immediate 2 s, 1.5 mA foot-shock and their brain tissue collected for *Egr-1* assays. Immediate foot-shock training again increased gene expression in both experimental groups relative to the HC (Figure 5.4). However, in contrast to preexposure day expression, mPFC *Egr-1* levels in Group Pre were significantly elevated above Group Alt-pre. This effect was observed in animals given a single 5 min exposure (Asok et al., 2013) or multiple-exposures (see Chapter 6; Schreiber et al., 2014) revealing a “stair-step” pattern of expression from HC, to non-associative control (Alt-Pre) to fear conditioned groups (Pre). When post-training *Egr-1* levels were examined in adult rats, similar preexposure and training day patterns were observed with non-differential increases following preexposure and a learning-related increase following foot-shock training observed only in the mPFC (Chakraborty et al., 2016).

The similarities in gene expression observed between adolescent (PD 31) and adult rats parallel behavioral results from our lab that show similar conditioning in these two ages during the CPFE (Robinson-Drummer & Stanton, 2015). Importantly, the CPFE emerges between PD 17 and PD 24 with freezing levels at test not differing between PD 24, PD31, or adult rats (Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011) which suggests that molecular processes associated with the CPFE may be fully developed by PD 24 although patterns at younger ages (e.g. PD 17) may be different. This hypothesis will be tested in greater detail in Chapter 7.

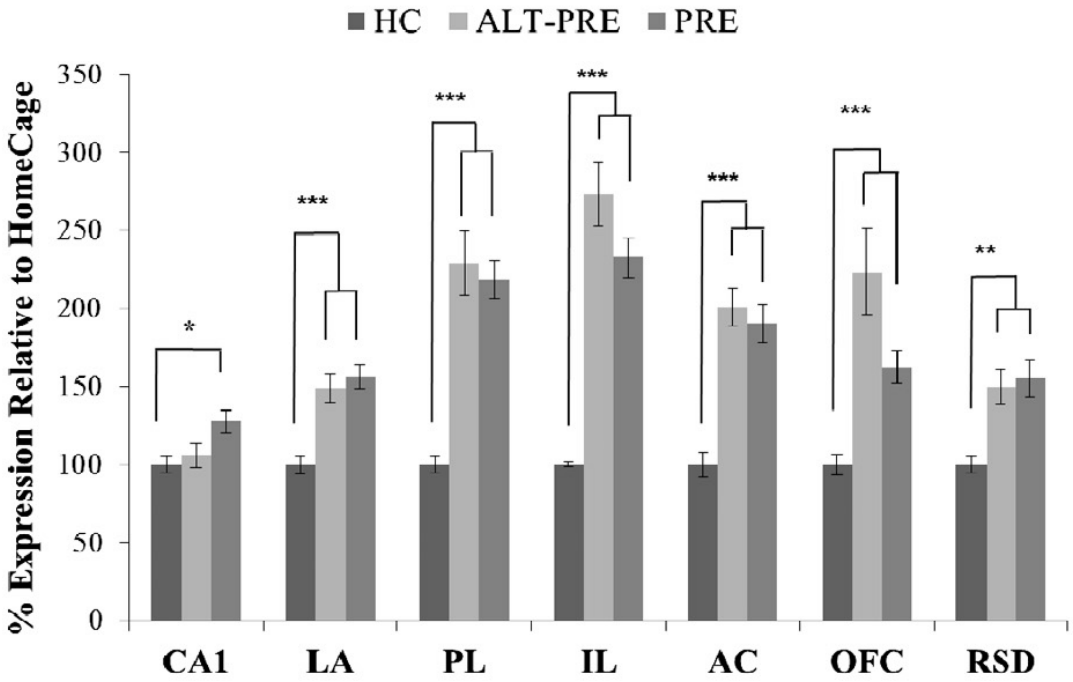

Figure 5.3 Mean (± SEM).percent expression of *Egr-1* mRNA compared to HC following context preexposure in the CPFE paradigm. Animals were preexposed to the training context (Group PRE) or an alternate context (Group ALT-PRE). Pre and Alt-Pre animals displayed significantly elevated *Egr-1* mRNA expression in all areas except for CA1 when compared to HC. Only Group PRE had increased *Egr-1* in CA1 compared to HC. $p < .05$, $p < .01$, $p < .001$. Figure taken from Asok et al. (2013).

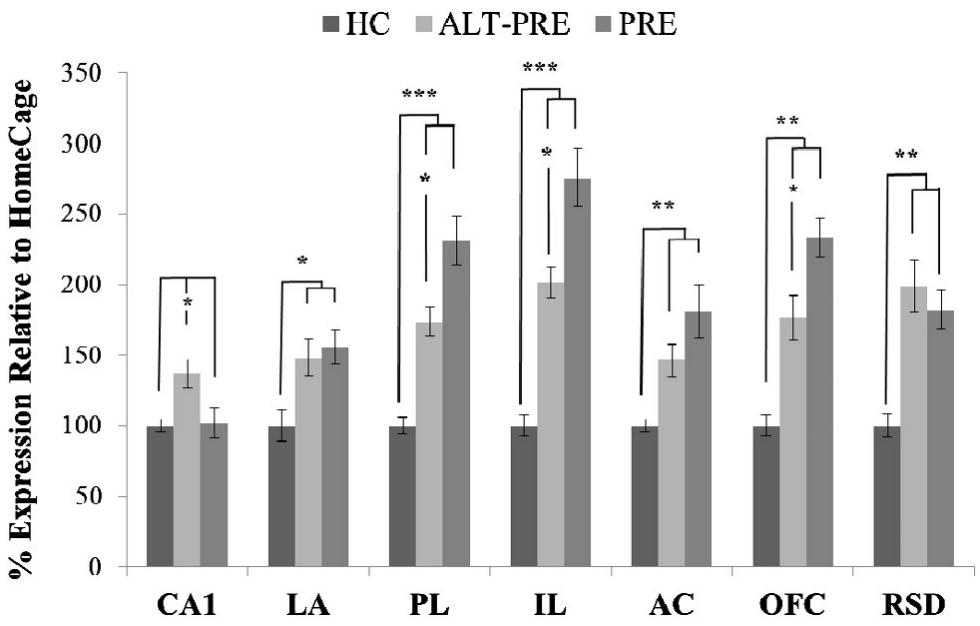

Figure 5.4 Mean (± SEM) percent expression of *Egr-1* mRNA compared to HC following immediate-shock training in the CPFE paradigm. Animals were preexposed on PD 31 and trained with an immediate foot-shock 24 hr later. PRE animals displayed significantly higher *Egr-1* mRNA than ALT-PRE animals in the PL, IL, and OFC. $p < .05$, $p < .01$, $p < .001$.

5.3 Effects of Hippocampal Muscarinic-type Acetylcholine Receptor Antagonism on the CPFE in Adolescent Rats

The use of fear conditioning to examine the pharmacological substrates of learning and memory has revealed a crucial role of central cholinergic function in memory. In adult rats, scopolamine, an antagonist to the muscarinic-type acetylcholine receptor (mAChr), selectively disrupts hippocampus-dependent Pavlovian conditioning, including background context fear conditioning (sCFC), while sparing simultaneously acquired fear conditioning to hippocampus-independent cue conditioning (Anagnostaras, Maren, & Fanselow, 1995; Anagnostaras, Maren, Sage, Goodrich, & Fanselow, 1999; Gale, Anagnostaras, & Fanselow, 2001; Hunt & Richardson, 2007). Similar to sCFC experiments, scopolamine administered systemically prior to the preexposure day of the CPFE impairs contextual fear conditioning in adult mice (Brown, Kennard, Sherer, Comalli, & Woodruff-Pak, 2011). In adult rats, administering intra-hippocampal scopolamine immediately after immediate-shock training (but not after preexposure) disrupts retention of context fear in the CPFE (Chang & Liang, 2012). The following study manipulated pre-conditioning mAChr activity, first systemically and then specifically in the hippocampus, during different phases of the CPFE to determine which component(s) of contextual fear conditioning requires cholinergic function (Robinson-Drummer et al., 2016).

In the first experiment, we systemically injected PD 31 rats with saline vehicle or 0.5 mg/kg scopolamine hydrobromide in saline, a mAChr antagonist, prior to preexposure (Group Pre), training (Group Train), or testing (Group Test), which were separated by 24 hr. A fourth group received injections of sterile saline (Group Sal) repeatedly across all three days to serve as a control for the injections. Figure 5.5

summarizes the findings from this manipulation. All animals that received scopolamine, regardless of the day of drug administration, showed reduced freezing on the test day compared to saline controls.

This outcome suggested a necessity of cholinergic function for learning processes (i.e. context learning, context-shock learning, and retrieval of contextual fear) that are engaged on each of the three experimental phases. Previous reports demonstrated a similar necessity of hippocampal neural activity across all three phases of the CPFE (Matus-Amat et al., 2004) so the next three experiments examined the effects of hippocampal cholinergic function during only single conditioning phases. Subjects were surgically implanted with bilateral cannulas which terminated above the CA1 subfield of the dorsal hippocampus (see Robinson-Drummer et al., 2016). An additional group of animals remained un-cannulated as an undisturbed (UND) comparison group. Intracranial infusions delivered 0.5 μL of phosphate buffered saline (PBS) or 35 μg of scopolamine in PBS per side per animal. Drug groups were preexposed to the training context (Context A) or an alternate context (Context B) to control for non-associative effects of conditioning. Representative results for these experiments are depicted in Figure 5.6. Similar to the systemic data, regardless of infusion day, antagonism of hippocampal mAChr prior to CPFE conditioning disrupted contextual fear conditioning which suggests that effects observed following systemic injections can be accounted for by antagonism of mAChr specifically in the hippocampus.

The previously described results suggest a major contribution of hippocampal cholinergic function to several learning and memory processes during fear conditioning. Preexposure day antagonism disrupts context encoding (but not

consolidation; see Chang & Liang, 2012) while training day effects could reflect deficits in retrieval of the context representation, or acquisition or consolidation of the context-shock association. Indeed, post-training scopolamine impairs retention of conditioned freezing, suggesting interference with context-shock memory consolidation (Chang & Liang, 2012). So the current training day effects most parsimoniously reflect lasting effects of pre-training drug infusion on consolidation rather than fear association *per se*. Testing-day results suggest intra-hippocampal scopolamine impaired retrieval or expression of either the context representation or of contextual fear memory.

Although hippocampal cholinergic function is clearly crucial for successful fear conditioning during the CPFE, the hippocampus is not the only fear-related region innervated by cholinergic projections. As noted in Chapter 2, the hippocampus is not the sole substrate of contextual fear conditioning and likely shares this role with the mPFC. Additionally, the mPFC receives a rich cholinergic projection (Armstrong, Saper, Levey, Wainer, & Terry, 1983; Henny & Jones, 2008; Luiten, Gaykema, Traber, & Spencer, 1987) and the role of this projection system in the mPFC during contextual fear conditioning remains largely unexplored.

Using a similar protocol as previously described (Robinson-Drummer et al., 2016) our lab utilized the CPFE to explore the contributions of the mPFC to contextual fear condition. Using the multiple preexposure protocol (Dokovna et al., 2013; Heroux, Robinson-Drummer, Rosen, & Stanton, 2016; Matus-Amat et al., 2004; Robinson-Drummer & Stanton, 2015) we infused 0.25 µL of PBS or 0.5 µg muscimol , a $GABA_A$ receptor agonist, dissolved in PBS directly in the mPFC prior to preexposure, training, or testing (Heroux et al., 2017). Similar to the effects of intra-

hippocampal scopolamine observed by Robinson-Drummer et al. (2016), mPFC inactivation prior to each conditioning phase disrupted contextual conditioning during the CPFE. These results confirm a functional role of the mPFC in the CPFE and lay the foundation for Chapter 9, which examines the role of mPFC cholinergic function in the CPFE.

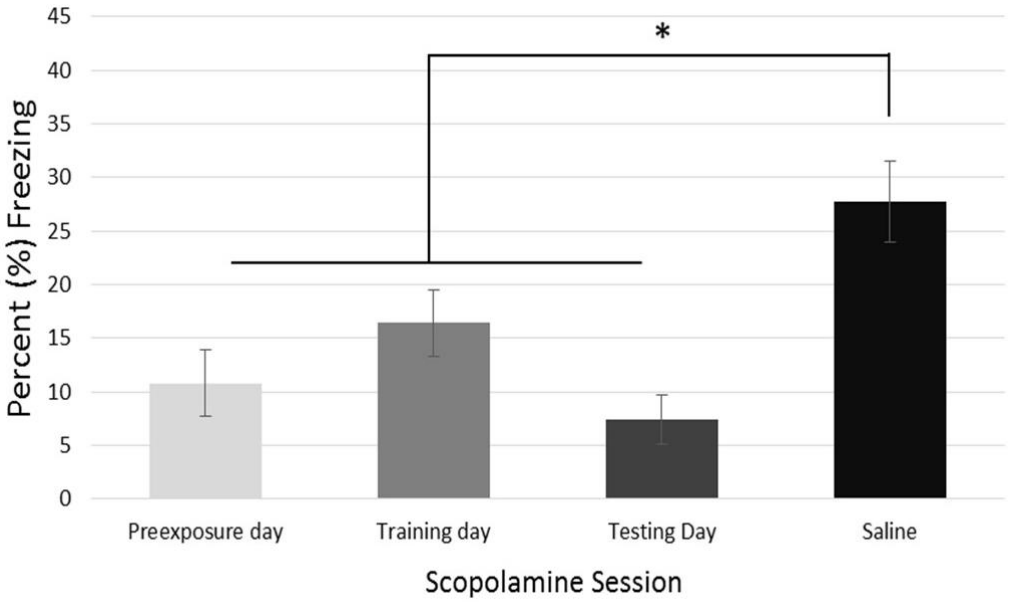

Figure 5.5 Mean (±SEM) percent test freezing as a function of group (Sal, Pre, Train, or Test). All animals were preexposed to the training and testing context on preexposure day. The Sal group significantly differed from all other groups ($p < 0.01$). Overall, scopolamine impaired the CPFE regardless of the day of administration. Image taken from Robinson-Drummer et al. (2016).

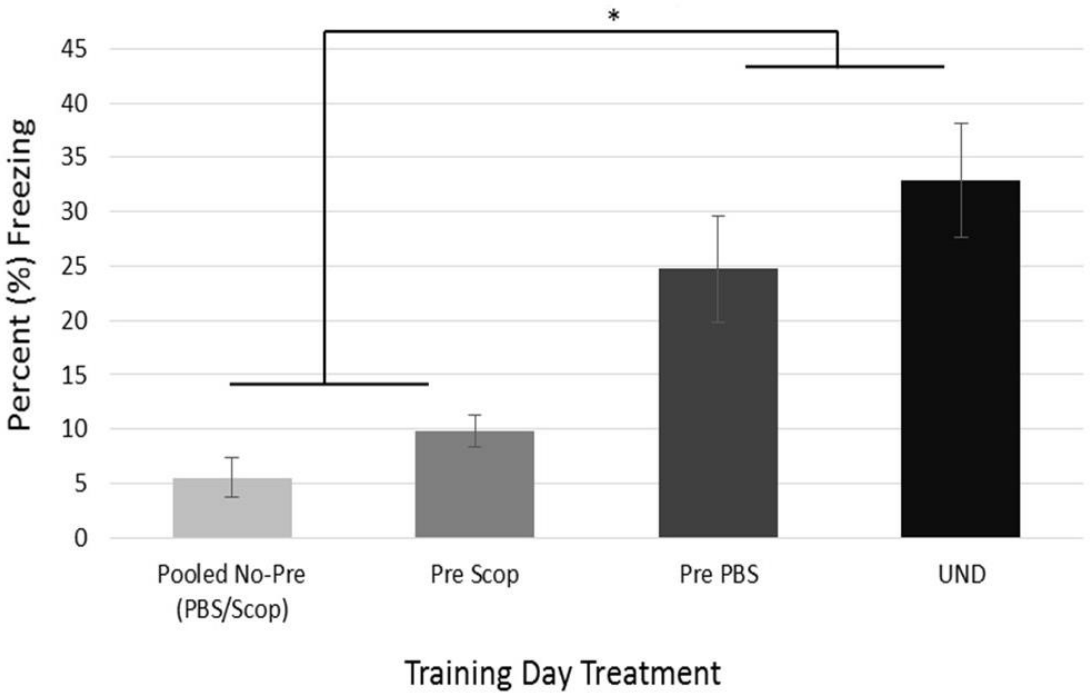

Figure 5.6 Mean (± SEM) percent test freezing as a function of training day injection group (No-Pre [a.k.a Alt-Pre], Pre-Scop, Pre-PBS and Pre-UND). Pre animals (Pre-Scop, Pre-PBS and Pre-UND) were preexposed to the training and testing context on preexposure day. The Pre-UND and Pre-PBS groups were significantly different from the (pooled) No-Pre and Pre-Scop ($p < 0.05$). The same pattern of results was obtained when intra-hippocampal scopolamine was administered only on the preexposure or testing days or across all three phases of the CPFE (data not shown). Thus, scopolamine impaired the CPFE when administered intra-hippocampally before any phase of the CPFE. Image taken from Robinson-Drummer et al. (2016).

5.4 Conclusions

The experiments reviewed above were fundamental to the hypotheses tested in the coming experimental chapters. First, the capacity for contextual memory retention does not emerge until after PD 17 and develops further after PD 31 (Robinson-Drummer & Stanton, 2015). Second, *Egr-1* expression is induced in many brain regions following novel context exposure, when the context representation is being formed. However learning-related patterns of expression are only observed in the mPFC following immediate foot-shock training (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014). Finally, hippocampal cholinergic function is critical for all phases of conditioning during the CPFE in adolescent rats (Robinson-Drummer et al., 2016) but the role of cholinergic function in mPFC remains to be tested. These studies establish a framework for the experimental chapters in this dissertation. Before turning to these, General Procedures are first considered in the next chapter.

Chapter 6

GENERAL PROCEDURES

The present chapter describes general procedures that are common to all experiments in this dissertation. Procedures that are unique to a particular experiment are provided within that experimental chapter. The descriptions provided here are based on those presented in previous publications (Asok et al., 2013; Dokovna et al., 2013; Jablonski & Stanton, 2014; Robinson-Drummer et al., 2016; Robinson-Drummer & Stanton, 2015; Schreiber et al., 2014).

6.1 Material and Methods

6.1.1 Subjects

Time-mated Long-Evans female rats were housed with breeder males overnight and were examined for an ejaculatory plug the following day and, if found, that day was designated as gestational day (GD) 0. Pregnant dams were housed in clear polypropylene cages measuring 45 x 24 x 21 cm with standard bedding and access to ad libitum water and rat chow. Animals were maintained on a 12:12 h light/dark cycle with lights on at 7:00 a.m. Date of birth (GD 22) was designated as postnatal day (PD) 0. Litters were culled on PD 3 to eight pups (usually four males and four females) and were paw-marked with subcutaneous injections of non-toxic black ink for identification. Pups were weaned from their dam on PD 21 (except where noted, see Chapters 7) and housed with same-sex litter mates in 45 x 24 x 17 cm cages. Two days prior to the start of conditioning, weaned animals were individually housed in small white polypropylene cages (24 x 18 x13 cm) with ad libitum access to water and rat chow for the remainder of the experiment. All subjects were treated in

accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Delaware following guidelines established by the National Institute of Health.

6.1.1.1 Alcohol Dosing

The general intubation procedure has been described in detail previously by Kelly and Lawrence (2008). Rat pups were randomly assigned to one of two exposure groups: sham-intubated (SI) or alcohol-exposed (EtOH). Alcohol was suspended in a custom milk solution (see Kelly & Lawrence, 2008) and delivered via intragrastric intubation at a dose of 5.25 g/kg/day. The following procedure for PD 7-9 alcohol exposure has been previously described (Brown, Calizo, Goodlett, & Stanton, 2007; Brown, Calizo, & Stanton, 2008; Jablonski & Stanton, 2014; Murawski & Stanton, 2011). Intubation typically began between 8:00 and 10:00 a.m. and pups were separated from their dams for approximately 15 min per dosing session. On PD 7, all pups were removed from their dams and placed in anti-static weigh boats over a low-setting heating pad to maintain the pups' core temperature. For alcohol-exposed pups, alcohol was delivered in the custom milk formula in a single-binge dose. The milk formula was delivered in a volume of 0.02778 mL/g body weight at 5.25 g/kg (23.80% v/v; EtOH group).

All pups were weighed prior to the first intubation of the dosing day. The intubation process began with first attaching PE10 tubing to a 1 mL syringe and coating the tubing with corn oil before gently passing the tubing down the esophagus into the stomach. For Group EtOH, the alcohol formula was released through the tube slowly over approximately 10 s. Group SI received the identical intubation process across 10 s however no formula was delivered. Following intubation of the final pup,

all pups were returned to their dam and the entire litter was returned to the colony room. Approximately 2 hr (± 10 min) after the first alcohol dose, pups were again separated from the dam for a second dosing session however prior to this second intubation, pups received a small tail-clip from which blood samples were collected with a capillary tube. Samples collected from the group SI were discarded and samples from group EtOH were stored for further analysis (see 'Blood Alcohol Concentration Analysis'). The second dosing session was identical to the first; however, pups from group EtOH received an infusion of milk formula only, without alcohol, in order to maintain proper weight gain (Jablonski & Stanton, 2014; M. D. Marino, Aksenov, & Kelly, 2004; Murawski & Stanton, 2010, 2011; Tran & Kelly, 2003). Pups from group SI again received a sham intubation (i.e. no formula administered). A third milk-only dose or sham intubation occurred 2 hr (± 10 min) following the second dosing session. Dosing on PD 8 through PD 9 was identical to that on PD 7 except no blood samples were collected and only one additional milk-only dose was given following the first daily alcohol administration.

6.1.1.2 Blood Alcohol Concentration Analysis

Blood samples collected on PD 7 from alcohol-exposed pups were centrifuged and the plasma was collected and stored at -20 °C. Blood alcohol concentrations (BACs) were determined using an Analox GL5 Analyzer (Analox Instruments, Luneburg, MA). Briefly, the rate of oxidation of alcohol in each plasma sample was measured and BACs (mg/dL) were calculated by comparing this rate to that of an alcohol standard solution with a known concentration.

6.1.2 Behavioral Apparatus and Stimuli

Fear conditioning occurred in four clear Plexiglas chambers, designated as Context A, as described previously (Heroux et al., 2016; Murawski & Stanton, 2010; Robinson-Drummer et al., 2016). The chambers measured 16.5 x 12.1 x 21.6 cm and were arranged in a 2 x 2 formation on a Plexiglas stand within a fume hood which provided ambient light and background noise. Each chamber had a grid floor made of 9 stainless steel bars, 0.5 cm in diameter and spaced 1.25 cm apart. The unconditioned stimulus (US), a 1.5 mA, 2 s foot-shock (except where noted, see Chapter 9), was delivered using a shock scrambler (Med Associates, Georgia, VT ENV-414S) connected to the grid floor. Video of each session (preexposure, training, testing) was recorded using FreezeFrame software (Actimetrics, Wilmette IL), which measures change in pixilation. Context B consisted of the same Plexiglas chambers used in Context A with modifications, which have been described previously (Asok et al., 2013; Murawski & Stanton, 2010; Robinson-Drummer et al., 2016; Schreiber et al., 2014). Wire mesh inserts, which protruded into the chambers, changed both the texture of the floor and the dimensions of chamber. In addition, white opaque coverings were added such that only the wall facing the camera remained unobscured.

6.1.3 Behavioral Procedures

Subjects were fear conditioned using the context preexposure facilitation effect (CPFE) training protocol. The CPFE consists of three conditioning phases (preexposure, training and testing) separated by 24 (±1) hr (see Chapter 1, Figure 1.1). The following section will describe the general procedures, however exceptions and specific experimental manipulations (e.g. age at exposure, variations in brain collection procedures, etc.) will be described in subsequent experimental chapters.

6.1.3.1 Preexposure

All behavioral procedures occurred during the light cycle. On the preexposure day animals were removed from their home cages, weighed and transported in groups of four to the waiting area outside of the conditioning room. Each conditioning chamber was thoroughly cleaned with a 5% ammonium hydroxide solution immediately prior to the conditioning trial. During context preexposure animals experienced either the single-exposure or multiple-exposure protocol. The single exposure protocol consisted of free chamber exploration for 5 min in either Context A or Context B. In the multiple-exposure protocol (Dokovna et al., 2013; Matus-Amat et al., 2004; Robinson-Drummer et al., 2016; Robinson-Drummer & Stanton, 2015), following the initial 5 min preexposure period, animals were removed and placed back in their transport boxes for approximately 1 min, after which they were returned to the chambers for a 1 min preexposure. This cycle was repeated four additional times yielding a total of five 1 min preexposures. After preexposure, animals were returned, in their respective transport boxes, to their home cages.

6.1.3.2 Training

Twenty-four (±1) hr after preexposure, animals were again weighed, placed into transport boxes and transported four at a time to the waiting area outside of the conditioning room. Following cleaning with 5% ammonium hydroxide, all animals were individually brought into the conditioning room and received an immediate (< 5s placement-to-shock interval) 2 s, 1.5 mA foot-shock upon placement into Context A. Animals were immediately removed, returned to their transport cages, and returned to their home cage.

6.1.3.3 Retention Testing

Twenty-four (±1) hr after immediate-shock training, animals were transported and placed into Context A in an identical manner to training day except no shock was delivered and freezing was recorded over a 5 min testing period.

6.1.3.4 Behavioral Data Statistical Analysis

The behavioral measure of fear learning analyzed for this dissertation was freezing, a species typical behavioral response to threat (Blanchard & Blanchard, 1969; Fanselow, 1986). Data were recorded and analyzed using FreezeFrame software (Actimetrics, Wilmette, IL) which measures change in pixilation. Video of each session (preexposure, training, testing) was recorded using FreezeFrame, with freezing defined as a bout of 0.75 s or longer without a change in pixels. Freezing was defined as the cessation of all visible movement except for respiration and video from the four chambers was simultaneously recorded. The freezing thresholds, initially set by Freezeframe for each subject, were examined by a human observer blind to the conditions of the subject. Adjustments were made as necessary to ensure exclusion of small motor movements mistakenly identified as freezing. We have validated this procedure against other scoring methods (e.g., hand scoring of video records by blind observers) and found that it is very reliable ($r = 0.976$; $p < .01$; unpublished observations). Freezing was analyzed as the percent (%) time spent freezing during the 5 min testing phase. Outliers were removed from test day freezing scores to reduce statistical variability. They were defined a priori as having a score ±1.96 standard deviations from the mean of all other rats in their respective groups. Usually no more than 1 data point per group was removed. Outliers were removed without knowledge of, or regard for, how their removal might affect subsequent statistical analyses. One-

way and factorial ANOVA's were used to statistically determine treatment effects on behavior (see experimental chapters below). Planned comparisons or post-hoc Newman-Keuls tests were used to analyze significant ANOVA effects. Statistical significance was set to $p < 0.05$.

6.1.4 *In situ* hybridization

This section will describe the general protocols used for brain extraction, *in situ* hybridization of tissue, image capture and analysis for Chapters 7 and 8. Specific experimental design and procedures will be further described in the experimental chapters.

6.1.4.1 Brain Collection

Detailed brain collection and *In situ* hybridization procedures were the same as previously described (Asok et al., 2013; Schreiber et al., 2014). During conditioning, all animals were sacrificed 30 (±3) min following chamber removal on the preexposure or training day. Homecage (HC) animals remained undisturbed in their home cages and were sacrificed while their littermate counterparts were undergoing conditioning. On the preexposure day, HC animals were completely naïve. However, on the training day HC controls were comprised equally of animals exposed to Context A or Context B once (Experiment 7.1 and 8.1) or after multiple-exposures (Experiment 7.2 and 7.3), to control for prior experience of the other experimental groups. Rats were sacrificed by rapid decapitation, and brains were immediately removed and frozen in 45 ºC isopentane and stored at 80 ºC until sectioned. Sixteen micrometer frozen coronal brain sections corresponding to the medial prefrontal cortex [mPFC; anterior cingulate cortex (AC), prelimbic cortex (PL), infralimbic

cortex (IL), orbitofrontal cortex (OFC)], dorsal hippocampus (dHPC) and lateral amygdala (LA) were sectioned on a cryostat (Leica Inc., Deerfield, IL) using the Paxinos and Watson stereotaxic rat brain atlas as a guide (Paxinos & Watson, 2013).

6.1.4.2 *In situ* hybridization

For *in situ* hybridization, an antisense RNA probe (riboprobe) was transcribed from a plasmid containing a sense cDNA sequence coding for a 230 bp sequence of *Egr-1* (gift from J. Milbrandt, Washington University, St. Louis, MO). The transcribed riboprobe incorporated a radioactively labeled ^{35}S UTP (approximately 1 x 106 dpm) using a T3 RNA polymerase Maxiscript kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY). After hybridization and washing, the dry slides were exposed to Kodak Biomax MR Film for two days and autoradiograms were analyzed for mean density values.

6.1.4.3 Image Analysis

Autoradiograms were captured and digitized to 8-bit gray values. Using ImageJ, the background was subtracted (2D-rolling ball radius of 50.0 pixels) and the mean density (mean gray value) for mRNA labeling was measured for each specific region on a brain slice. A ^{14}C standard with known amounts of radioactivity was exposed and captured with each film. The standard was then used to create a 3rd degree polynomial equation to convert the mean gray values of each slide to nCi/g. The nCi/g values for animals in each experiment were then normalized against (expressed as a ratio of) the average nCi/g of the home cage animals for that experiment and in the specified region of interest to obtain a proportionate score of the home cage group which equaled 100%. When nCi/g scores fell ±1.96 standard

deviations from the proportionate group mean for a region, that score was defined as an outlier and was excluded from further analysis (typically no more than 1 score/group/region was excluded, see results of individual experiments). In one instance (Exp. 7.1), variability in nCi/g scores across assays within the same study required that outliers be removed based on HC ratio (for each assay) rather than nCi/g scores (pooled across assays).

6.1.4.4 Statistical Analysis

Statistica 13 (Statsoft, Tulsa, Oklahoma) was used for all statistical analyses. Statistical analyses was as described previously (Asok et al., 2013; Murawski & Stanton, 2011; Schiffino et al., 2011; Schreiber et al., 2014). For *in situ* hybridization data, comparisons between normalized data in the experimental groups (i.e. Pre and Alt-Pre groups) and the Homecage controls were assesses by one-way or factorial ANOVA. Post hoc Dunnett's or Newman-Keuls test were used to assess any significant main effects revealed by the ANOVA.

Chapter 7

AGE AND EXPERIENCE DEPENDENT CHANGES IN EGR-1 EXPRESSION DURING THE ONTOGENY OF THE CONTEXT PREEXPOSURE FACILITATION EFFECT (CPFE)

This chapter was recently reviewed for publication, revised and resubmitted (Robinson-Drummer, Chakraborty, Heroux, Rosen, & Stanton, under review). As noted previously, the hippocampus, amygdala and mPFC are important for contextual fear conditioning and for CPFE performance (Chapters 2 & 5). In addition, changes in *Egr-1* expression in these regions on the preexposure- and training days of the CPFE have been studied in adolescent (PD31) and adult rats (Figures 5.3 and 5.4; Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014). The CPFE develops between PD17 and 24 (Chapter 3, Figure 3.4) but it is currently unknown whether maturation of regional molecular activity correlates with the ontogenetic profile of the CPFE. The current chapter characterizes the ontogenetic profile of *Egr-1* expression in the mPFC, amygdala and hippocampus across phases of the CPFE at PD 17, 24, and 31 in order to identify molecular processes that may contribute to the emergence of contextual fear conditioning in developing animals.

7.1 Experiment 7.1: *Egr-1* Expression in Developing Rats Following a Single Context Exposure or Immediate Foot-shock Training

The current experiment characterized the changes in *Egr-1* mRNA expression in the mPFC, hippocampus and amygdala of developing rats in response to context preexposure or immediate foot-shock training. We hypothesized that gene expression patterns would change between PD 17 and PD 24 with the latter displaying adolescent- and adult-like gene expression patterns. In contrast, PD 17 rats, which do not exhibit the CPFE, would show different patterns of expression following

conditioning correlating with their lack of fear conditioning. Specifically, the prediction was that *Egr-1* activation on the preexposure day, or learning-related changes in expression observed in several prefrontal subregions on the training day, would be absent in PD 17 animals but not PD24 rats.

Experiment 7.1 was comprised of four sub-experiments---behavior (7.1a), preexposure-day *Egr-1* expression (7.1b), and training-day *Egr-1* expression (7.1c and 7.1d). The first three sub-experiments compared PD17 vs. PD24 rats. The fourth sub-experiment (7.1d) examined only PD31 rats. This last experiment was added after the outcome of the other sub-experiments was known, in order to replicate (within the present study) behavior and training-day *Egr-1* expression from a previous report involving PD31 rats (Asok et al., 2013). In addition to *Egr-1* expression (Exp. 7.1d), behavior from PD31 rats was added (as a separate analysis) to Experiment 7.1a.

7.1.1 Materials and Methods

7.1.1.1 Subjects

Animal husbandry was as described in our previous reports (Chapter 6) in accordance with the NIH guidelines and protocols approved by the Institutional Animal Care and Use Committee at the University of Delaware. Animals beginning conditioning on PD 17 were group housed with their dams throughout the procedure. Animals beginning conditioning on PD 24 or PD 31 were weaned into group cages (45 x 24 x 17 cm) with 2-4 same-sex littermates on PD 21 and then individually housed into individual opaque white cages (45 x 24 x 17 cm) two days prior to experimental procedures and for the remainder of the study. No more than one same-sex littermate

was assigned to a given experimental condition (except on a few occasions where their data were averaged, noted below).

7.1.1.2 Apparatus, Stimulus and Procedure

Full details of the apparatus and stimuli were previously described in Chapter 6. Preexposure, training and testing occurred one day apart beginning on PD 17, PD 24 or PD 31. Animals were assigned to one of three groups: Group Pre (preexposed to Context A), Group Alt-Pre (preexposed to Context B) or home cage control (HC; no preexposure). HC controls sacrificed on the training day experienced context (equally Pre and Alt-pre context) preexposure on the previous day to control for the effects of prior behavioral experience on baseline gene expression. The current experiment utilized the single-exposure CPFE protocol which involved a single 5 min context exposure followed 24 hr later by an immediate 1.5 mA, 2 s foot-shock. Animals were sacrificed 30 (±3) min following chamber removal on either the preexposure day or training day for tissue collection, *in situ* hybridization of brain slices and *Egr-1* mRNA assessment (see section Chapter 6: *In situ* Hybridization). Another group of Pre and Alt-Pre littermates was not sacrificed so that they could undergo retention testing of conditioned fear 24 hr after immediate shock training.

Figure 7.1 contains representative diagrams (Figure 7.1a) and example brain slices with contrast enhancement (Figure 7.1b) to illustrate *in situ* hybridization results for regions in the mPFC (left) and the dHPC and LA (right). A schematic of the CPFE and animal sacrifice procedure is illustrated in Figure 7.1c.

7.1.1.3 Statistical Analysis

Statistica 13 (Statsoft, Tulsa, Oklahoma) was used for all statistical analyses. Statistical analyses was as described previously (Asok et al., 2013; Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011; Schreiber et al., 2014). All ANOVAs initially included sex as a factor. However, because main or interaction effects were rarely found (only 3 out of 48 comparisons), data are summarized and analyzed (Tables 7.1-7.3) collapsed across this variable. Exceptions were not replicated across experiments, were only found in single regions for specific ages and were not reflected in pair-wise comparisons so they are not reported in text. Gene expression data were analyzed separately for each region. To avoid excessive, repetitive statistical reporting in the text, the statistical findings for all gene-expression analyses are summarized in Tables 7.1-7.3.

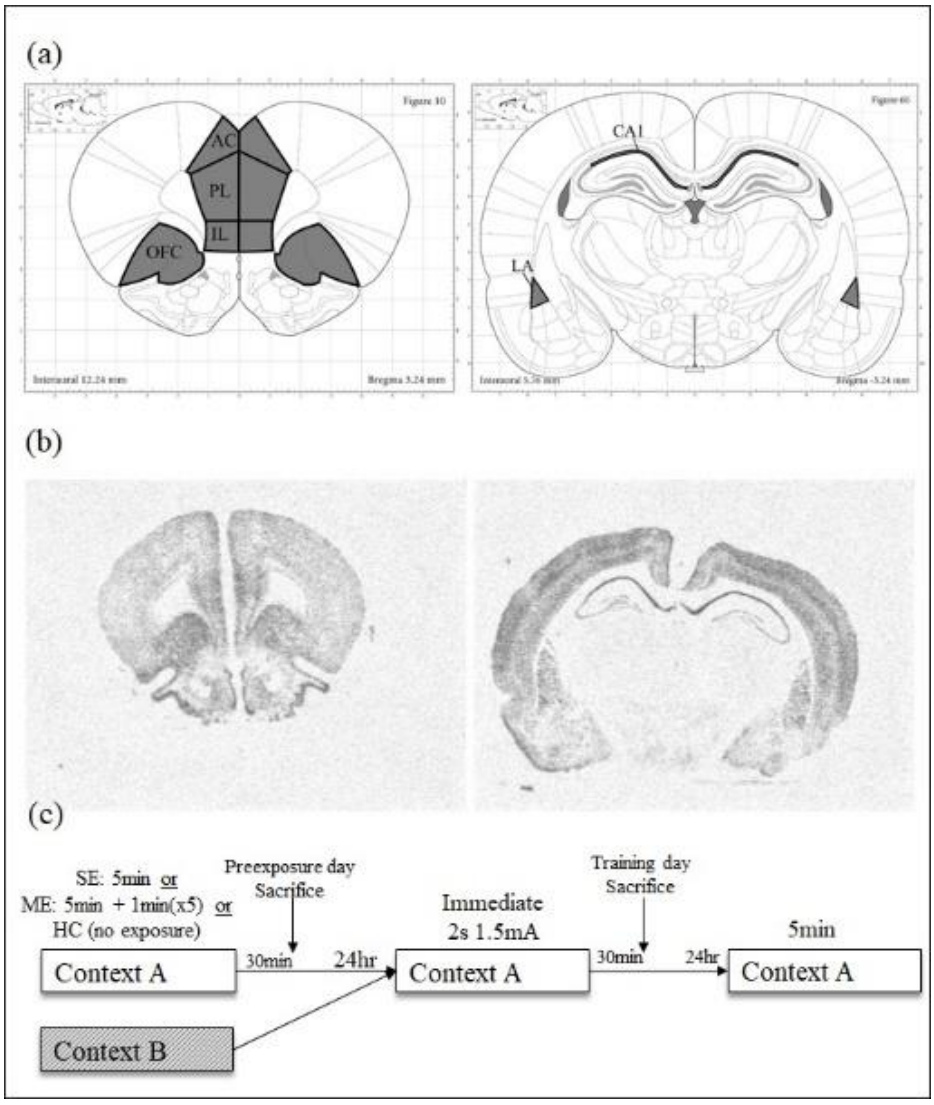

Figure 7.1 (a) Illustrations of brain regions analyzed following *in situ* hybridization. Regions included in *Egr-1* analysis are outlined in black and shaded in gray. Guides for CA1 and LA were obtained from plate 60 and guides for PL, IL, AC, and OFC were obtained from plate 10. Images are adapted from The Rat Brain in Stereotaxic Coordinates, 7th Ed (Paxinos & Watson, 2013). (b) Example brain slices with contrast enhancement to illustrate *in situ* hybridization results for regions in the mPFC (left), dHPC and LA (right). (c) Schematic of CPFE and animal sacrifice procedure illustrating the single–exposure (SE; Experiments 7.1 and 7.2) and multiple-exposure (ME; Experiments 7.2 and 7.3) protocols.

7.1.2 Experiment 7.1a: Single-exposure CPFE Behavioral Results

Behavior subjects were 25 and 22 animals from 20 litters preexposed on PD 17 or PD 24, respectively, to either the training context (Group Pre) or the alternate context (Group Alt-Pre). This yielded a four-group experimental design (PD 17 Pre, PD 17 Alt-Pre, PD 24 Pre and PD 24 Alt-Pre). Three total outliers were removed (PD 17 Alt-Pre n = 1; PD 17 Pre n = 1; PD 24 Alt-Pre n = 1) and statistical analyses were carried out on the remaining 44 animals with final group sizes as follows: (PD 17 Alt-Pre n = 12; PD 17 Pre n = 10; PD 24 Alt-Pre n = 11; PD 24 Pre n = 11). In addition, 25 animals from 10 litters were preexposed at PD 31. One case of same-sex littermate oversampling was averaged in the PD31 Alt-Pre group and two outliers were removed (PD 31 Pre n = 1, PD 31 Alt-Pre n = 1) leaving 11 final animals in the PD 31 Alt-Pre and PD 31 Pre groups.

Figure 7.2a shows the behavioral results for Experiment 7.1a. Comparing PD 17 and PD 24 animals (Figure 7.2a; left panel), a 2 (Age: PD 17 v PD 24) x 2 (Preexposure condition: Pre v Alt-Pre) factorial ANOVA revealed a significant effect of age [$F(1,40) = 7.04$, $p = .01$], preexposure condition [$F(1,40) = 15.68$, $p < .001$] and a age x preexposure condition interaction [$F(1,40) = 10.75$, $p < .01$]. Newman-Keuls post-hoc analyses revealed that group PD 24 Pre froze significantly more during retention testing than group PD 24 Alt-Pre, PD 17 Pre and the PD 17 Alt-Pre groups (all p's < .001) however these groups did not differ (p's > .62). In addition, the CPFE was present at PD 31 as confirmed by a significant effect of group [$F(1, 20) = 56.90$, $p < .001$] in a one-way ANOVA (Figure 7.2a, right panel). These results replicate previous findings from our lab (Asok et al., 2013; Jablonski et al., 2012; Murawski & Stanton, 2011; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011) that PD 24

and PD 31 but not PD 17 rats display 24 hr retention of contextual fear conditioning when trained on the single-exposure CPFE protocol.

Experiment 7.1b: Single-Exposure CPFE Preexposure day *Egr-1* expression PD17 and PD24									
	AC			PL			IL		
	n (HC, Alt-Pre, Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)		
PD17	13, 11, 12			13, 11, 11			13, 12, 12		
PD24	12, 10**, 11			12, 10**, 13*			12, 10***, 12**		
	F	*p*		*F*	*p*		*F*	*p*	
Age	14.36	< .001	PD24 > PD17	15.63	<.001	PD24 > PD17	14.63	<.001	PD24 > PD17
Preexposure	4.15	0.02	Alt-Pre > HC	4.92	0.01	Pre, Alt-Pre > HC	16.66	<.001	Pre, Alt-Pre > HC
Age x Preexposure	3.88	0.03	PD 24 Pre, Alt-Pre > PD 17, HC	4.18	0.02	PD 24 Pre, Alt-Pre > PD 17, HC	4.04	0.02	PD 24 Pre, Alt-Pre > PD 17, HC
	OFC			dHPC			dLA		
	n (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Alt-Pre, Pre)		
PD17	13, 12, 11			11, 12, 12			12, 12, 12		
PD24	11, 11***, 12**			12, 10, 11**			12, 11***, 10***		
	F	*p*		*F*	*p*		*F*	*p*	
Age	15.57	<.001	PD24 > PD17	8.04	<.01	PD24 > PD17	20.6	<.001	PD24 > PD17
Preexposure	8.74	<.001	Pre, Alt-Pre > HC	4.84	0.01	Pre, Alt-Pre > HC	8.27	<.001	Pre, Alt-Pre > HC
Age x Preexposure	4.89	0.01	PD 24 Pre, Alt-Pre > PD 17, HC	3.14	0.0502	*NS*	6.03	<.01	PD 24 Pre, Alt-Pre > PD 17, HC

Experiment 7.1c: Single-Exposure CPFE Training day *Egr-1* expression PD17 and PD24									
	AC			PL			IL		
	n (HC, Alt-Pre, Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)		
PD17	13, 12**, 11**			13, 12***, 11***			13, 12***, 11**		
PD24	12, 12**, 10**			12, 10***, 11**			12, 10***, 11***		
	F	*p*		*F*	*p*		*F*	*p*	
Age	0.18	0.68	*NS*	0.55	0.46	*NS*	9.39	<.01	PD24 > PD27
Preexposure	6.24	<.01	Pre, Alt-Pre > HC	20.94	<.001	Pre, Alt-Pre > HC	16.12	<.001	Pre, Alt-Pre > HC
Age x Preexposure	0.23	0.8	*NS*	1.54	0.22	*NS*	2.46	0.09	*NS*
	OFC			dHPC			dLA		
	n (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Alt-Pre, Pre)		
PD17	13, 13***, 11**			12, 12***, 9*			12, 10***, 11***		
PD24	12, 10***, 11**			12, 10***, 11*			12, 11**, 12		
	F	*p*		*F*	*p*		*F*	*p*	
Age	0.47	0.49	*NS*	1.54	0.22	*NS*	0.99	0.32	*NS*
Preexposure	11.50	<.001	Pre, Alt-Pre > HC	15.72	<.001	Alt-Pre > Pre > HC	13.37	<.001	Alt-Pre > Pre > HC
Age x Preexposure	0.15	0.86	*NS*	0.45	0.64	*NS*	1.52	0.23	*NS*

Experiment 7.1d: Single-Exposure CPFE Training day *Egr-1* expression PD31									
	AC			PL			IL		
	F	*p*	*n* (HC, Alt-Pre, Pre)	*F*	*p*	*n* (HC, Pre, Alt-Pre)	*F*	*p*	*n* (HC, Pre, Alt-Pre)
PD 31	7.08	< .01	12, 12**, 11**	12.02	< .001	12, 12*, 12***	13.32	< .001	12, 12**, 12***
Preexposure			Pre, Alt-Pre > HC			Pre > Alt-Pre >HC			Pre > Alt-Pre >HC
	OFC			dHPC			dLA		
	F	*p*	*n* (HC, Alt-Pre, Pre)	*F*	*p*	*n* (HC, Pre, Alt-Pre)	*F*	*p*	*n* (HC, Pre, Alt-Pre)
PD 31	3.80	0.03	12, 12*, 12*	2.87	0.069	11, 12, 12	3.04	0.059	11, 12, 12
Preexposure			Pre, Alt-Pre > HC						

Table 7.1 Statistical results (*F* and *p* values) and group numbers (*n)* for Experiment 7.1. Factorial ANOVA and Newman-Keuls post-hoc tests were used to evaluate changes in gene expression as a function of age at preexposure (using the single-exposure protocol). Gene expression was analyzed in PD 17 and PD 24 animals following preexposure (Experiment 7.1b) and training (Experiment 7.1c) and in PD31 animals following training (Experiment 7.1d). Asterisks (next to group *n*) represent Newman-Keuls post hoc tests comparing experimental groups to homecage (HC) controls. * $p < .05$, ** $p < .01$, *** $p < .001$. Main or interaction effects are described by inequality signs (> or <) and non-significant effects are listed as "NS."

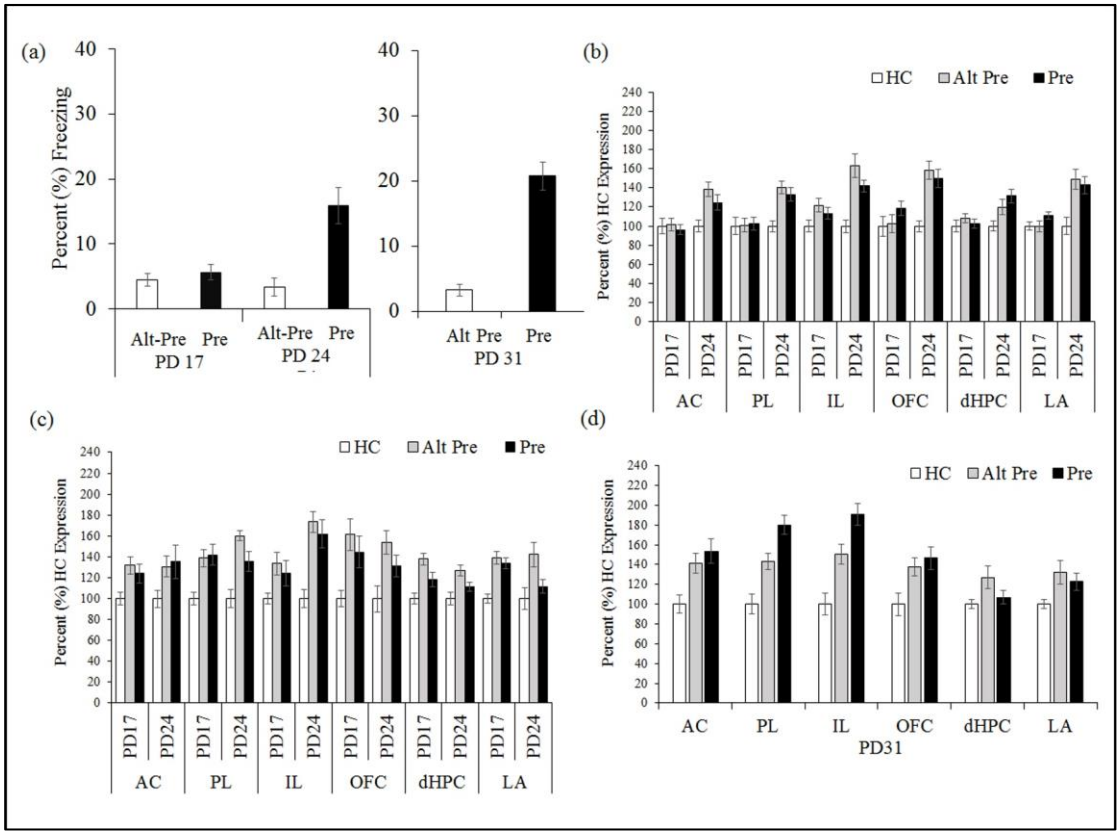

Figure 7.2 Experiment 7.1 (a) Mean (±SEM) percent test freezing 24 hr after immediate shock training in rats preexposed on PD 17, PD 24 or PD 31 using the single-exposure (SE) CPFE protocol. The CPFE was evident in animals preexposed on PD 24-31 but not PD 17, as reflected in higher freezing relative to Alt-Pre controls. (b) Mean (±SEM) percent expression of *Egr-1* mRNA relative to homecage (HC) controls following context preexposure. Pre and Alt-Pre rats preexposed on PD 24 displayed significantly elevated gene expression in all regions relative to HC controls and to rats preexposed on PD 17. These increases were absent in rats preexposed on PD 17. (c) Following immediate foot-shock training, Pre and Alt-Pre expression was increased similarly between PD 17 and PD 24 rats in all regions except the IL where PD 24 expression was elevated relative to PD 17 expression, and LA where only Alt-Pre exceeded HC in PD 24 rats. (d) Mean (±SEM) percent expression of *Egr-1* mRNA in PD 31 rats. Learning-related increases in gene expression (Pre > Alt-Pre) in the mPFC were evident in the PL and IL but no other regions.

7.1.3 Experiment 7.1b: Exposure to a Novel Context Drives *Egr-1* Expression in Juvenile but not Infant Rats

The current experiment assessed developmental differences in *Egr-1* expression in the mPFC, dHPC and LA following the preexposure day of the CPFE to determine the potential contribution of this gene expression to the developmental emergence of the CPFE. Subjects began the behavioral protocol on either PD 17 or PD 24. Using 13 litters, tissue was collected from 39 animals preexposed on PD 17 and 37 animals preexposed on PD 24. Groups HC, Alt-Pre and Pre were initially comprised of 13 animals and, after outliers were removed (see Chapter 6), final group sizes ranged from 10-13 animals (Table 7.1).

Figure 7.2b shows the results of Experiment 7.1b. In general, novel context exposure increased gene expression in all regions on PD 24 but not PD 17. This was true for both Context A (Group Pre) and Context B (Group Alt-Pre) which did not differ but were both elevated over HC control levels on PD 24 but not PD 17. In all regions (see Table 7.1, Exp 7.1b), separate 2 (Age: PD 17 v PD 24) x 3 (Preexposure condition: HC, Alt-Pre, Pre) factorial ANOVAs revealed significant main effects of age, preexposure condition and significant age x preexposure condition interactions (except in the dHPC where the interaction was only trending; $p = .0502$). Newman-Keul's post-hoc tests revealed that, regardless of preexposure condition, PD 24 animals had significantly elevated *Egr-1* expression following context preexposure relative to PD 17 animals (p's $< .01$). Further examination of the interaction effects revealed that for the mPFC (AC, PL and IL) and the LA both group Pre and Alt Pre showed significantly increased *Egr-1* expression relative to group HC when animals were preexposed at PD 24 (p's $< .02$) but there was no increase observed at PD 17 (p's $> .05$). Trends were similar in the dHPC even though the interaction failed to reach

significance [$F(2, 62) = 3.14$, $p = .0502$]. Together, these results confirm that exposure to a novel context significantly increases relative *Egr-1* expression in all regions on PD24 but does not drive gene expression on PD 17.

7.1.4 Experiment 7.1c: Post-Training Day *Egr-1* in PD 17 and 24 Rats

Experiment 7.1c assessed ontogenetic differences in *Egr-1* expression following the training day of the CPFE in PD 17 and PD 24 rats (Figure 7.2c). Tissue was collected from 39 animals (13 litters) and 36 animals (12 litters) from the PD 17 and PD 24 age groups, respectively. After outliers were removed, statistics were run on each region separately with final group sizes ranging from 9-13 animals (see Table 7.1, Exp 7.1c). In contrast to Experiment 7.1b, 2 (Age: PD 17 v PD 24) x 3 (Preexposure condition: HC, Alt-Pre, Pre) followed by Newman-Keul's post-hoc test did not reveal any main effects of age or age x preexposure interactions in any region (p's > .22; see Table 7.1) except in the IL where there was a main effect of age ($p <$ .01) reflecting increased relative expression in the PD 24 animals compared to PD 17. There was a significant main effect of preexposure condition in all regions, regardless of age, such that groups Pre and Alt Pre had increased *Egr-1* expression relative to HC (p's < .02). These results confirm that immediate foot-shock training can drive *Egr-1* in the mPFC, dHPC and LA in both PD 17 and PD 24 animals although only PD 24 animals acquire the CPFE.

7.1.5 Experiment 7.1d: Post-Training Day *Egr-1* in PD31 Rats

In Experiment 7.1c, PD 24 animals did not show the learning-related changes in mPFC gene expression following foot-shock training as previously observed at PD 31 (Asok et al., 2013). Historically these animals have not differed in there levels of conditioning (Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011) so these results were surprising. Because we have observed this effect using the single-exposure protocol on PD 31 in only a single study (Asok et al., 2013), Experiment 7.1d sought to assess the replicability of this effect by reexamining PD 31 gene expression following the single-exposure protocol. Tissue was collected on the training day from 39 animals from 10 litters. After outliers were removed, statistics were run on each region separately with final group sizes ranging from 11-13 (see Table 7.1, Exp 7.1d).

Figure 7.2d shows the results of Experiment 7.1d. One-way ANOVA (Preexposure: HC, Alt-Pre, Pre) revealed a significant main effect of Preexposure condition in all four prefrontal regions (Table 7.1). Newman-Keul's test showed both experimental groups were significantly increased (all p's < .05) relative to HC. In the AC and OFC, Newman Keuls post-hoc test showed no significant difference between Pre and Alt-Pre gene expression (p's > .41). In contrast, in both the PL and IL, Pre group expression was significantly elevated above Alt-Pre and HC, replicating the learning-related change in PD31 rats previously reported with the single-exposure protocol by our lab (Asok et al., 2013).

In the LA and dHPC, there was a marginally significant main effect of group with a trending increase in both Pre and Alt-Pre gene expression above HC. Although not significant, these trends are similar to previous findings from our lab using both single-exposure (Asok et al., 2013) and multiple-exposure (Schreiber et al., 2014)

CPFE protocols in PD31 rats. These results, together with Experiments 7.1b and 7.1c, suggest *Egr-1* expression induced by novel context exposure and foot-shock training during the CPFE develops between PD 17, 24 and PD 31.

7.2 Experiment 7.2: Training Day *Egr-1* Expression in PD 24 Rats Following Single- or Multiple-context Preexposure

The behavioral results of Experiment 7.1 revealed relatively low conditioning (~15%) in the PD 24 animals (Figure 7.2A) relative to our historical data (~25-30%; Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011). The lack of learning-related expression (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014) in the PD 24 group may reflect a true ontogenetic difference between young and older rats however it is also possible that weak conditioning in the PD 24 animals simply did not drive learning-related *Egr-1* expression. In order to address this possibility, Experiment 7.2 manipulated the amount of exposure received on the preexposure day in order to strengthen conditioning on the training day. The effect of single- vs. multiple-preexposure protocols on behavior (Experiment 7.2a) and training-day gene expression (Experiment 7.2b) was examined in PD 24 rats.

7.2.1 Methods and Materials

7.2.1.1 Subjects

Animals began conditioning on PD 24 (±1 day). Pups were weaned into group cages (45 x 24 x 17 cm3) with 2-4 same-sex littermates on PD 21 and then individually housed into individual opaque white cages (45 x 24 x 17 cm3) two days prior to experimental procedures (PD 22) and for the remainder of the study. No more than one same-sex littermate was assigned to a given experimental condition.

7.2.1.2 Apparatus, Stimuli, and Procedure

The current experiment compared *Egr-1* mRNA expression following the single-exposure (SE) or multiple-exposure (ME) CPFE protocol as described in Chapter 6 (see Behavioral Procedures). Both preexposure conditions were followed 24

hr later by immediate foot-shock training. Animals were sacrificed 30 (±1) min following chamber removal on the training day for tissue collection and *in situ* hybridization of brain regions (see section Chapter 6: *In Situ* Hybridization). HC controls (comprised equally of rats preexposed to the Pre and Alt-pre context) experienced context preexposure on the previous day but were not conditioned on the training day. Another group of Pre and Alt-Pre littermates were not sacrificed so that they could undergo retention testing of conditioned fear 24 hr after immediate shock training.

7.2.1.3 Statistical Analysis

Analyses were as described in Chapter 6 and Experiment 7.1 and are summarized in Table 7.2.

7.2.2 Experiment 7.2a: PD 24 Single- vs Multiple-exposure CPFE Behavioral Results

Behavioral subjects were 36 animals from 15 litters with the remaining littermates used during conditioning for *in situ* hybridization. On the preexposure day, animals received either the single-exposure (SE) or the multiple-exposure (ME) CPFE protocol which has been described previously (Chapter 6; Dokovna et al., 2013; Robinson-Drummer & Stanton, 2015; Rudy & Wright-Hardesty, 2005). This yielded a four-group design (SE Pre, SE Alt-Pre, ME Pre and ME Alt-Pre). Four outliers were removed (ME Alt-Pre $n = 1$; ME Pre $n = 1$; SE Alt-Pre $n = 1$; SE Pre $n = 1$) and final group sizes were as follows: (ME Alt-Pre $n = 4$; ME Pre $n = 5$; SE Alt-Pre $n = 12$; SE Pre $n = 11$).

Figure 7.3a shows the behavioral results for Experiment 7.2a. A 2 (Exposures: Single v Multiple) x 2 (Preexposure condition: Pre v Alt Pre) factorial ANOVA

revealed a significant effect of preexposure condition [$F(1,27) = 10.53$, $p < .01$], but no main effect of exposure [$F(1,27) = .46$, $p = .50$] or exposure x preexposure condition interaction [$F(1,27) = .05$, $p = .82$]. Newman-Keuls post-hoc analyses revealed significantly higher freezing in group Pre (regardless of number of exposures) relative to the Alt-Pre groups (p's < .01). The increased freezing in the Pre groups relative to the Alt-Pre group indicates the presence of the CPFE however the additional exposure in the ME Pre group did not increase overall conditioned freezing over the SE Pre group. That group SE Pre froze more than its counterpart in Experiment 7.1 (~25% vs. 15%) reflects cross-experiment variation (sampling error) that we have occasionally seen in our previous studies.

7.2.3 Experiment 7.2b: Post-Training Day *Egr-1* Comparing Single- and Multiple-preexposures in PD 24 Rats

The effect of single vs. multiple context preexposure on *Egr-1* expression following immediate foot-shock on the training day in PD 24 rats is shown in Fig. 7.3b. Subjects began the behavioral protocol on PD 24 (59 animals from 14 litters). For final group sizes see Table 7.2. In general, immediate-shock training elevated *Egr-1* expression over HC control levels in all exposure groups and in all regions except dHPC (Figure 7.3b), however learning-related changes were observed in some prefrontal sub-regions following the ME but not SE protocol.

Separate 2 (Exposure: Single v Multiple) x 3 (Pre condition: HC, Pre, Alt Pre) factorial ANOVAs (see Table 7.2) revealed a main effect of exposure (p's < .02) in the AC, PL, IL, OFC and dHPC [but not the LA ($p = .34$] such that, regardless of preexposure condition, animals in the group ME showed increased *Egr-1* expression relative to group SE except in the OFC and dHPC where this pattern was reversed.

Additionally, a significant main effect of preexposure condition (p's < .001) was found in all mPFC subregions and the LA [but not the dHPC (p = .45)] such that groups Pre and Alt pre, regardless of exposure, had significantly elevated *Egr-1* above HC. The AC and IL also had significant exposure x pre condition interactions (p's < .05). Both single and multiple preexposures increased *Egr*-1 expression in groups Pre and Alt Pre relative HC, however group multiple-exposure Pre (ME Pre) had gene expression significantly elevated above all other experimental groups which did not differ from each other (p's > .88). A similar non-significant trend was also observed in the PL (Figure 7.3b).

Experiment 7.2 revealed learning-related *Egr-1* expression on the training day in the AC and IL mPFC (but not the dHPC or LA) following the multiple-exposure but not the single-exposure protocol. This result was not found in PD 31 rats as both the single-exposure (Asok et al., 2013) and multiple-exposure (Schreiber et al., 2014) protocol produce learning-related increases in some mPFC sub-regions. These results suggests that more context training is needed at PD 24 to achieve similar patterns of mPFC activation as that observed at older ages.

Experiment 7.2b: Single v Multiple- Exposure CPFE Training day *Egr-1* expression PD24									
	AC			PL			IL		
	n (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Alt-Pre, Pre)		
SE PD 24	3, 10, 8			3, 10***, 8***			3, 10, 7		
ME PD 24	4, 8**, 8***			3 , 8***,7***			3, 7**, 8***		
	F	*p*		*F*	*p*		*F*	*p*	
Exposure	10.07	< .01	ME > SE	5.72	0.02	ME > SE	8.77	<.01	ME > SE
Pre Condition	12.55	<.001	Pre, Alt-Pre > HC	12.27	<.001	Pre, Alt-Pre > HC	15.70	<.001	Pre > Alt-Pre > HC
Exposure x Pre Condition	3.28	0.049	ME Pre > all others	1.57	0.22	*NS*	4.74	0.02	ME Pre > all others
	OFC			dHPC			dLA		
	n (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Alt-Pre, Pre)		
SE PD 24	3, 9***, 7***			5, 9, 6			4, 10***, 5***		
ME PD 24	3, 8,*** 8***			3, 7, 8			5, 8***, 9***		
	F	*p*		*F*	*p*		*F*	*p*	
Exposure	5.38	0.03	SE >ME	6.23	0.02	SE >ME	0.94	0.34	*NS*
Pre Condition	11.43	<.001	Pre > Alt-Pre > HC	0.82	0.45	*NS*	9.03	< . 001	Pre, Alt-Pre > HC
Exposure x Pre Condition	2.66	0.09	*NS*	1.54	0.23	*NS*	2.2	0.13	*NS*

Table 7.2 Statistical results (*F* and *p* values) and group numbers (*n)* for Experiment 7.2b. Factorial ANOVA and Newman-Keuls post-hoc tests were used to evaluate changes in PD 24 gene expression following immediate shock training as a function of context exposures on the preexposure day (single vs multiple exposures). Asterisks (next to group *n*) represent Newman-Keuls post hoc tests comparing experimental groups to homecage (HC) controls. * $p < .05$, ** $p < .01$, *** $p < .001$. Main or interaction effects are described by inequality signs (> or <) and non-significant effects are listed as "NS."

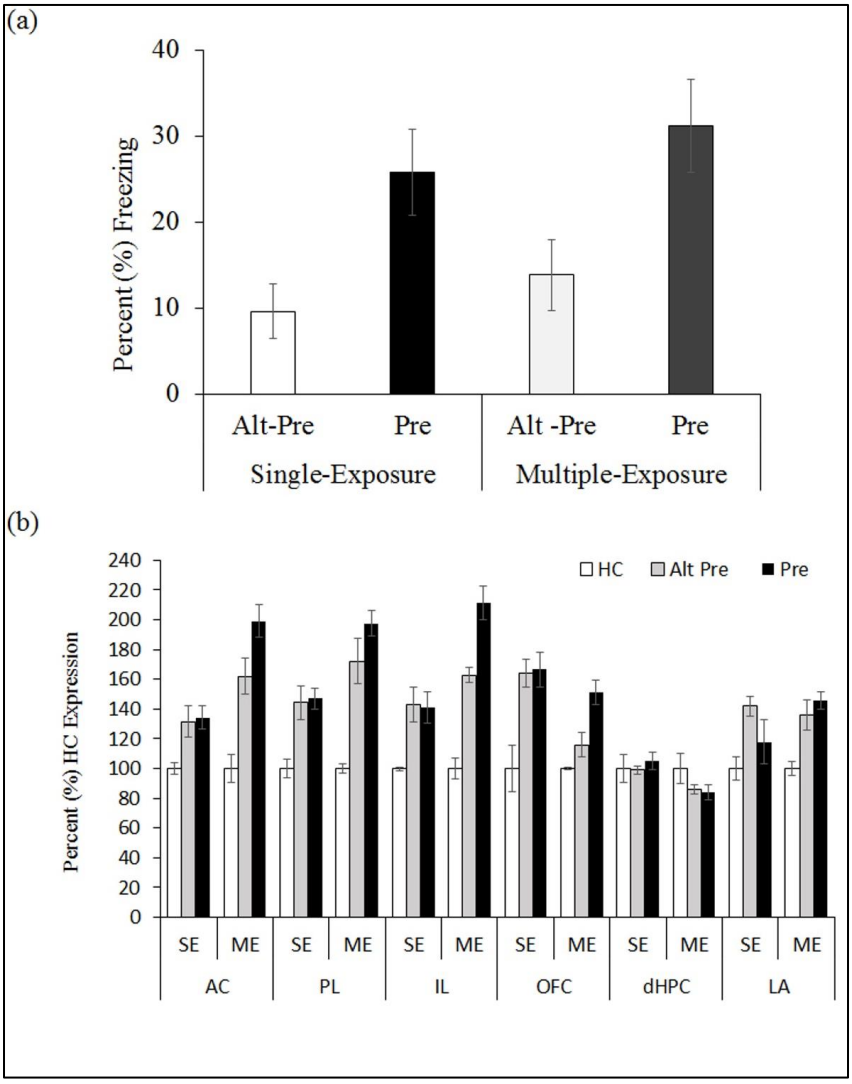

Figure 7.3 Experiment 7.2 (a) Mean (±SEM) percent test freezing 24 hr after immediate shock training in rats preexposed on PD 24 using the single- (SE) or multiple-exposure (ME) CPFE protocol. A significant CPFE was evident using both protocols with no difference observed between SE and ME freezing and both groups were significantly elevated over Alt-Pre controls. (b) Mean (± SEM) percent expression of *Egr-1* mRNA relative to homecage controls following immediate shock training. Immediate shock training increased *Egr-1* expression in Pre and Alt-Pre rats relative to HC controls using both the SE and ME protocols. However a learning-related increase (Pre > Alt-Pre) in the IL and OFC was only observed using the ME protocol.

7.3 Experiment 7.3: Training Day *Egr-1* Expression in PD 17 and PD 24 Rats Following the Multiple-exposure Preexposure Day Protocol

The different patterns of mPFC gene expression following the single- vs. multiple-exposure protocols was interesting in light of no behavioral differences observed across these protocols. To replicate this finding and examine if this same change would be observed in PD 17 rats, gene expression was again measured following immediate foot-shock training using a multiple-exposure protocol on the preexposure day comparing PD 17 and PD 24 animals.

7.3.1 Materials and Methods

7.3.1.1 Subjects

Animal husbandry was as described in Experiment 7.1 (and Chapter 6).

7.3.1.2 Apparatus, Stimuli, and Procedure

Experimental procedures were as described in Experiment 7.1 except that the multiple-exposure (instead of the single-exposure) CPFE protocol was utilized. Animals were sacrificed 30 (±3) min following chamber removal on the training day for tissue collection and *in situ* hybridization of brain regions (see Chapter 6: *In Situ* Hybridization). Another group of Pre and Alt-Pre littermates were not sacrificed so that they could undergo retention testing of conditioned fear 24 hr after immediate shock training. Brain sections of rats sacrificed after training or home-cage controls were assessed for *Egr-1* expression.

7.3.1.3 Statistical Analyses

Analyses were as described in Experiment 7.1 and are summarized in Table 7.3.

7.3.2 Experiment 7.3a: Behavioral results for PD 17 and PD 24 rats following the multiple-exposure preexposure day protocol

Behavior subjects were 36 animals from 14 litters (the remaining littermates were used during conditioning for *in situ* hybridization). Two animals were removed as statistical outliers (PD 17 Pre $n = 1$; PD 24 Alt-Pre $n = 1$) and the final group sizes were as follows: (PD 17 Pre $n = 11$; PD 17 Alt-Pre $n = 6$; PD 24 Pre $n = 12$; PD 24 Alt-Pre $n = 5$). Figure 7.4a shows the behavioral results for Experiment 7.3. Similar to Experiment 7.1, 2 (Age: PD 17 v PD 24) x 2 (Preexposure Condition: Pre v Alt-Pre) factorial ANOVA revealed a significant effect of age [$F(1,30) = 4.39$, $p = .04$], preexposure condition [$F(1,30) = 9.98$, $p < .01$] and an age x preexposure condition interaction [$F(1,30) = 8.97$, $p < .01$]. Again, Newman-Keuls post-hoc analyses revealed significantly higher freezing in PD 24 Pre relative to groups PD 24 Alt-Pre, PD 17 Pre and the PD 17 Alt-Pre groups (all p's $< .01$) however the PD17 groups did not differ (p's $> .52$). Like Experiment 7.1, PD 17 animals failed to show a CPFE that is readily observed using the same parameters at PD 24, even with stronger conditioning (i.e. multiple-exposures).

7.3.3 Experiment 7.3b: Post-Training day *EGR-1* in PD 17 and PD 24 rats following the multiple-exposure preexposure day protocol

Gene expression in Experiment 7.3 was examined on the training day 24 hr following the same ME protocol used in Experiment 7.2. For both PD 17 and PD 24, 38 animals from 14 litters were sacrificed on the training day and final group sizes ranged from 10-13 animals (see Table 7.3).

Figure 7.4b shows the *Egr-1* results of Experiment 7.3 and Table 7.3 shows the results of separate 2 (Age: PD 17 v PD 24) x 3 (Pre condition: HC, Pre, Alt Pre) ANOVA for each region. No effects or interactions were found in the dHPC (p's >

.17), and in the LA, there was a main effect of preexposure condition ($p < .02$) reflecting groups Pre and Alt-Pre elevation over HC at both ages. ANOVA for the all mPFC subregions revealed significant main effects age, preexposure condition and significant age x preexposure condition interactions (p's < .03). Generally, groups Pre and Alt Pre were significantly elevated above HC for both ages (p's < .001). In the OFC, this effect was driven by significant increases in PD 24 Pre and Alt Pre above HC; for PD 17, Pre and Alt-Pre were not significantly elevated above HC. In the AC, both PD 24 and PD 17 experimental groups were elevated above HC but PD 24 expression was higher than PD 17 expression. For both the PL and the IL PD 24 *Egr-1* expression was elevated in groups Pre and Alt-Pre above HC and PD 17 with no significant increase observed in PD17 groups. Additionally, in both the PL and IL at PD 24 Group Pre was elevated above group Alt-Pre and HC.

For two prefrontal (PL and IL) subregions, PD 24 animals showed the learning-related increase in Group Pre gene expression (relative to Alt-Pre and both PD17 groups) observed in the AC and IL of Experiment 7.2 and in previous reports (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014). However, even with increased context exposure, PD 17 animals did not show learning-related increases in *Egr-1* expression and expression levels actually seemed lower in this experiment relative to that observed in Experiment 7.1. This may suggest that much of the gene expression observed in PD 17 animals on the training day following the single-exposure protocol may be due to novelty of the fear chambers which was reduced with multiple-exposures. Importantly, the positive effect at PD 24 in the IL replicates Experiment 7.2 while the effect in PL, which was only a trend in

Experiment 7.2, was significant in the current experiment and the effect in the AC, which is not significant here, was positive in Experiment 7.2. The multiple-exposure protocol did not change the ontogeny of contextual fear conditioning which was absent at PD 17 and robust at PD 24 nor did it produce learning-related changes in gene expression at PD 17 that were observed at older ages (Experiment 7.1d and 7.2; Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014).

Experiment 7.3b: Multiple-Exposure CPFE Training day *Egr-1* expression PD17 and PD24									
	AC			PL			IL		
	n (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Alt-Pre, Pre)		
PD17	13, 11*, 11*			11, 11, 10			11, 12*, 11		
PD24	12, 11***, 11***			11, 10***, 12***			11, 11***, 12***		
	F	*p*		*F*	*p*		*F*	*p*	
Age	14.73	<.001	PD24 > PD17	76.03	<.001	PD24 > PD17	39.66	<.001	PD24 > PD17
Preexposure	25.21	<.001	Pre, Alt-Pre > HC	27.56	<.001	Pre, Alt-Pre > HC	25.22	<.001	Pre, Alt-Pre > HC
Age x Preexposure	4.01	0.02	Pre 24, Alt Pre 24 > PD 17 >HC	26.45	<.001	Pre 24 > Alt Pre 24 > PD 17, HC	15.45	<.001	Pre 24 > Alt Pre 24 > PD 17, HC
	OFC			dHPC			LA		
	n (HC, Pre, Alt-Pre)			*n* (HC, Pre, Alt-Pre)			*n* (HC, Alt-Pre, Pre)		
PD17	12, 11, 11			12, 10, 12			12, 11*, 11*		
PD24	11, 12***, 12***			12, 11, 11			12, 12*, 11*		
	F	*p*		*F*	*p*		*F*	*p*	
Age	35.11	<.001	PD24 > PD17	1.91	0.17	*NS*	1.27	0.26	*NS*
Preexposure	20.19	<.001	Pre, Alt-Pre > HC	1.51	0.23	*NS*	4.29	0.02	Pre, Alt-Pre > HC
Age x Preexposure	9.55	<.001	Pre 24, Alt Pre 24 > PD 17 >HC	0.67	0.51	*NS*	0.35	0.7	*NS*

Table 7.3 Statistical results (*F* and *p* values) and group numbers (*n)* for Experiment 7.3b. Factorial ANOVA and Newman-Keuls post-hoc tests were used to evaluate changes in gene expression as a function of age at preexposure (using the multiple-exposure protocol). Gene expression was analyzed in PD 17 and PD 24 animals following immediate shock training. Asterisks (next to group *n*) represent Newman-Keuls post hoc tests comparing experimental groups to homecage (HC) controls. * $p < .05$, ** $p < .01$, *** $p < .001$. Main or interaction effects are described by inequality signs (> or <) and non-significant effects are listed as "NS."

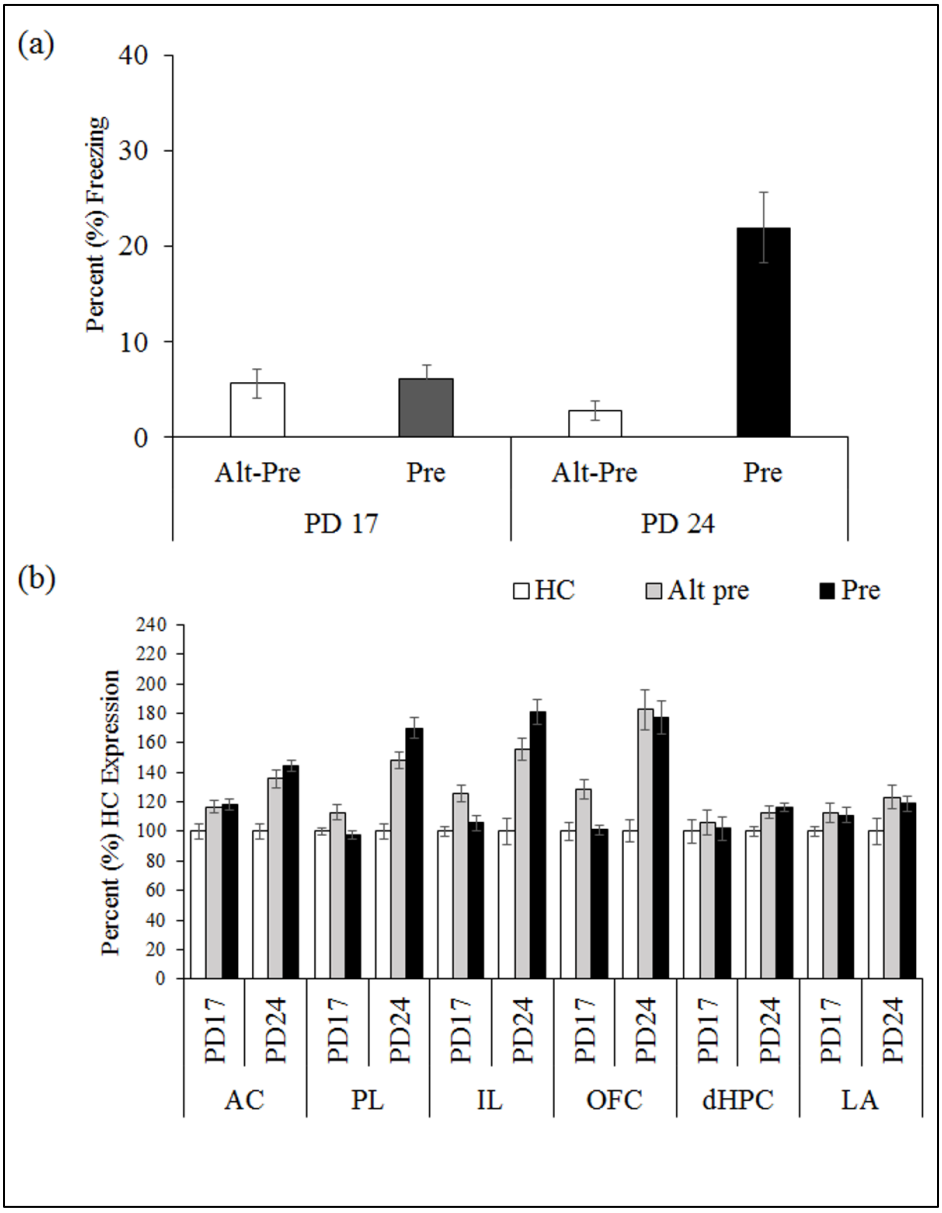

Figure 7.4 Experiment 7.3 (a) Mean (±SEM) percent test freezing 24 hr after immediate shock training in rats preexposed on PD 17 or PD 24 using the multiple-exposure (ME) CPFE protocol. The CPFE (Pre > Alt-Pre) was evident in animals preexposed on PD 24 but not PD 17. (b) Mean (±SEM) percent expression of *Egr-1* mRNA relative to homecage controls following immediate shock training. Learning-related increases (Pre > Alt-Pre) in gene expression were evident in the PL and IL subdivisions of mPFC on PD 24 but not on PD 17.

7.4 Summary of Findings

The previous experiments examined the ontogenetic changes in *Egr-1* mRNA expression during contextual fear learning using the CPFE. Similarly to that observed at older ages (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014), preexposure to the training context increased *Egr-1* expression in the mPFC, hippocampus and amygdala at PD 24 however this increase was absent at PD 17. However, following immediate shock training, *EGR-1* expression was increased similarly in the brain between both ages although neither showed the learning-related increase in mPFC gene expression observed at adolescence. When context exposure was increased on the preexposure day, PD 24 but not PD 17 rats displayed learning-related expression patterns in the mPFC. These results, particularly on the preexposure day, reveal a clear maturation of gene expression indicative of an ontogenetic change in molecular function that correlates with the emergence of the CPFE.

7.5 Discussion

7.5.1 Circuit Maturation and the Ontogeny of Context Learning/Memory in Developing Rats

The current report, in conjunction with previous studies from our lab, reveals a gradual increase in prefrontal *Egr-1* expression in response to context exposure between infancy and adulthood. Following context preexposure, adult levels of prefrontal *Egr-1* increased by over 200% in some regions relative to home-cage controls (Chakraborty et al., 2016) while adolescent expression (PD31) for all but one group did not increase above 150% (Asok et al., 2013). For PD 24 rats (Experiment 7.1b) IL and OFC expression increased by approximately 45-65% and AC and PL expression changes were even smaller, increasing by only 30-40%. Most interestingly,

PD 17 rats (Experiment 7.1b) showed no increase in *Egr-1* expression on the preexposure day. The gradual increase in *Egr-1* expression likely reflects both structural and functional maturation of the mPFC and its connections as the mPFC shows significant ontogenetic changes through young adulthood in the rat (Chapter 3; Ferguson & Gao, 2014; Van Eden & Uylings, 1985; Zhang, 2004). Activity in mPFC becomes integral for memory retrieval with increased time between memory encoding and retrieval (Frankland & Bontempi, 2005; Wiltgen & Tanaka, 2013). This activity also supports long-term memory consolidation processes, which function to preserve context memories in adult animals, but does not appear to maintain remote memories in developing rodents (for behavioral studies see Akers et al., 2012; Robinson-Drummer & Stanton, 2015; Rudy & Wright-Hardesty, 2005). Reduced mPFC activity at the time of acquisition may result in insufficient cortical reactivation by the mPFC at the time of retrieval. Likewise, immaturity of molecular activity in the mPFC would likely impair context learning and/or consolidation in infant rats as prefrontal function on the preexposure day is necessary for successful performance of the CPFE in PD31 rats (Heroux et al., 2017; Robinson-Drummer, Heroux, & Stanton, 2017).

The prelimbic and infralimbic cortices are thought to control fear expression and inhibition, respectively, however more recent research has also postulated a role for the mPFC in contextual fear learning (Giustino & Maren, 2015; Maren et al., 2013; Sierra-Mercado et al., 2011; Zelikowsky et al., 2013; Zelikowsky et al., 2014). Following context preexposure both regions respond to context exploration suggesting that they both participate in the initial encoding or consolidation of the context representation. Recent electrophysiological research has found evidence of context-specific activity in mPFC neuronal firing which may stabilize hippocampal context

representations (Kyd & Bilkey, 2003, 2005) and track multiple-exposures to the same context (Hyman et al., 2012). That the gene expression in the prelimbic and infralimbic cortices following foot-shock training were reflective of preexposure conditioning may indicate a temporal tracking of contextual experiences that functions to facilitate proper fear expression or inhibition when multiple context memories have been acquired (i.e. a neutral memory following preexposure vs a fear memory during training). Fear acquisition in the CPFE (post-shock freezing) does not require mPFC activity, however, 24 hr retention is impaired (Heroux et al., 2017). This suggests that if the mPFC is participating in disambiguating the context meaning following CPFE training it is doing so through consolidation processes that function to alter memories after the context-shock association happens. Elucidating whether *Egr-1* in the prefrontal cortex serves these learning functions requires further experimentation.

In contrast to the prefrontal cortex, *Egr-1* expression in the hippocampus was much smaller and more stable than in mPFC across ages in response to context exposure. Increases varied by about 25-50%, regardless of age, between PD 24 (Experiment 7.1b) and adulthood (Asok et al., 2013; Chakraborty et al., 2016) while PD 17 animals showed no increase (Experiment 7.1b). These results parallel the ontogeny of the CPFE in that PD 17 animals historically do not show the effect while a fully developed CPFE is observed beginning at PD 24 (Jablonski et al., 2012; Pugh & Rudy, 1996; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011). These results are of interest because a previous study which reduced hippocampal *Egr-1* using antisense during the CPFE in adult rats showed no effect of preexposure day infusions on testing day performance (J. L. Lee, 2010). It is possible that reducing *Egr-1* activity in the hippocampus is insufficient to reduce context learning due to

compensatory mechanisms in the mPFC. Using adult animals, Zelikowsky et al. (2013) showed that the mPFC can compensate for hippocampal damage during contextual fear conditioning, whereas if both regions are compromised contextual conditioning is lost. This suggests that boosting mPFC activity (alone or in conjunction with the hippocampus) in PD 17 animals may alleviate learning and/or consolidation deficits on the preexposure day. This is a potentially fruitful direction for future research.

Changes in amygdalar response to context exposure varied according to age at exposure. Exposure at PD 24 (Experiment 7.1b) and PD 31 (Asok et al., 2013) increased *Egr-1* expression while exposure at PD 17 (Experiment 7.1b) and adulthood (Chakraborty et al., 2016) did not. These results may reflect changes in communication between regions like the mPFC and amygdala during this developmental window. The post-weanling period is associated with dynamic changes in prefrontal-amygdala physiology, structure and functional connectivity (Arruda-Carvalho, Wu, Cummings, & Clem, 2017; Bouwmeester, Smits, & Van Ree, 2002; Bouwmeester, Wolterink, & van Ree, 2002; D. E. Ehrlich et al., 2013; D. E. Ehrlich et al., 2012; Ferguson & Gao, 2014; Van Eden & Uylings, 1985). Increases in experience-driven molecular activity in the LA could reflect increased amygdalar activity resulting from overactive mPFC excitatory afferents until local pruning reduces this activity in adulthood. Some evidence of an mPFC-amygdala maturational cycle has been observed in developing mice (Arruda-Carvalho et al., 2017) however whether a similar developmental profile exists in the rat remains to be determined.

7.5.2 Weanlings and Juveniles Display Age- and Training-dependent *Egr-1* Expression

The training day learning-related differences in *Egr-1* expression between Pre and Alt-Pre groups observed at older ages (Asok et al., 2013; Chakraborty et al., 2016) was not observed at either PD 17 or PD 24using the single-exposure protocol (Experiment 7.1c). This finding in PD 24 rats was surprising when considering that PD 24 and PD 31 rats show equivalent levels of context fear (Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015; Schiffino et al., 2011). Interestingly, additional exposures using the multiple-exposure protocol in PD 24 rats (Experiment 7.2b and 7.3b) produced learning-related increases in the prefrontal cortex similar to that observed in PD 31 and adult animals while PD 17 animals actually showed reduced expression in the Pre group with additional preexposure (Experiment 7.3b). The increase at PD 24 was mildly blunted relative to older animals in previous work (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014) which suggests that, like the preexposure day, training day *Egr-1* expression is still maturing at this age. In addition, these results suggest that to achieve similar patterns of relative cortical activation, younger animals need increased exposure to the training context on the preexposure day. Paradoxically, if learning-related changes in *Egr-1* expression are necessary for successful conditioning in the CPFE then we would predict similar levels of expression in the single- and multiple- exposure tasks at PD 24. However, the current finding of equivalent fear conditioning across single-exposure and multiple-exposure protocols on PD 24, even though only multiple-exposure produces learning-related changes in prefrontal *Egr-1* expression on the training day suggests this pattern of expression is not required for the CPFE, at least in PD 24 animals. Studies that

knock down *Egr-1* expression in mPFC during the single- and multiple-exposure protocols at PD 24 are needed to resolve these issues.

Few studies have explored the ontogenetic emergence of immediate early gene expression using infant and adolescent rats. Deal et al. (2016) measured *Egr-1* mRNA expression in several brain regions during fear conditioning. Interestingly, following acquisition of tone fear conditioning they found no increase, relative to homecage controls, in amygdalar or hippocampal *Egr-1* expression between rats trained on PD 17 or PD 24. These results are in contrast to results from standard contextual fear conditioning (sCFC) in adults (Chakraborty et al., 2016; Malkani & Rosen, 2001) and adolescents (Schreiber et al., 2014) where the LA of the amygdala showed increased expression following both delayed and immediate foot-shock training. Furthermore, during the CPFE in juveniles (Experiments 7.1c and 7.2b), adolescents (Experiment 7.1d; Asok et al., 2013; Schreiber et al., 2014) and adults (Chakraborty et al., 2016) training with an immediate foot-shock increased *Egr-1* expression in both preexposure conditions relative to homecage. There are several reasons why these results may not agree, including Deal's use of tone fear conditioning with background contextual conditioning and examination of *Egr-1* expression in the entire basolateral region of the amygdala instead of the dorsal lateral nucleus as in the present study. Further experimentation will be necessary to discern the causes of these conflicting findings.

7.5.3 *Egr-1:* A Molecular Component of Contextual Fear Acquisition and Consolidation?

Immediate early genes (IEGs) participate in the consolidation of memories partly through the maintenance of post-training synaptic plasticity. *Egr-1* knockout mice exhibit increased decay of long-term potentiation (LTP) and long-term memory

deficits while early LTP and short-term memory is preserved (Jones et al., 2001). When considering the fear circuit, impaired learning and memory is associated with reduced IEG expression (including *Egr-1*) in the amygdala (Maddox et al., 2011; Malkani & Rosen, 2001; Malkani et al., 2004), hippocampus (Farina & Commins, 2016; J. L. Lee, 2010; J. L. Lee, Everitt, & Thomas, 2004) and mPFC (Farina & Commins, 2016). Taken together, these studies illustrate a functional role for *Egr-1* activity during conditioning across several different paradigms and in several regions. The current results suggest this role for *Egr-1* expression particularly on the preexposure day when *Egr-1* in the mPFC or dHPC may be facilitating plasticity-dependent consolidation of the context representation. Again, experiments that address the causal role of *Egr-1* (i.e. knock down of *Egr-1* expression) are required to address this issue. Other plasticity-related genes (e.g. *Arc*, *c-Fos*, *BDNF*, etc) may also show protracted expression in response to conditioning across development, a hypothesis we have begun to investigate (Heroux et al., 2018).

7.5.4 Conclusions

The role of *Egr-1* as a "marker" for neuronal plasticity is less debated than its function as a component of associative learning and memory consolidation/reconsolidation (Alberini, 2009, 2011; S. Davis, Bozon, & Laroche, 2003; Okuno, 2011; Rosen, 2004), however its role in learning across development is still poorly understood. Here, we examined the ontogenetic profile of *Egr-1* expression in infant and juvenile rodents using the CPFE and found significant maturation of gene expression that was region- and training-phase specific. Our findings suggest that differences in regional expression likely reflect ongoing development of cortical and subcortical structures necessary for conditioning. They also indicate the emergence of

novelty-induced activity in regions crucial for contextual conditioning. In addition, we've found that learning-related expression of mPFC *Egr-1* can be dissociated from the ontogeny of conditioned freezing during the CPFE. However, this IEG may nevertheless serve as a molecular component of other psychological processes necessary for learning. Assessment of the causal role of *Egr-1* within specific brain regions in the ontogeny of learning is a fruitful area for further experimentation.

Chapter 8

IMPAIRMENT OF THE CONTEXT PRE-EXPOSURE FACILITATION EFFECT IN ADOLESCENT RATS BY NEONATAL ALCOHOL EXPOSURE IS ASSOCIATED WITH DECREASED EGR-1 MRNA EXPRESSION IN THE PREFRONTAL CORTEX

The previous chapter demonstrated the emergence of age- and experience-dependent changes in *Egr-1* mRNA expression following conditioning during the CPFE. In normally developing adolescent rats (PD 31), mPFC activity reliably correlates with learning in terms of the relative gene expression observed following context-shock association (i.e. immediate shock training; Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014). The following experiment examines changes in *Egr-1* expression in relation to impaired behavioral expression of the CPFE using a rodent model of Fetal Alcohol Spectrum Disorder (FASD; Chapter 4; Dokovna et al., 2013; Jablonski & Stanton, 2014; Murawski et al., 2012; Murawski & Stanton, 2010, 2011).

8.1 Experiment 8.1: Impaired CPFE in FASD Rats is Associated with Decreased mRNA Expression in Prefrontal Sub-regions

Egr-1 mRNA expression was examined in the mPFC [prelimbic (PL) and infralimbic (IL) cortices], CA1 subfield of the hippocampus (CA1) and lateral nucleus of the amygdala (LA) of rats neonatally exposed to alcohol. Gene expression was assessed following immediate foot-shock training and we hypothesized that gene expression patterns would be diminished in FASD rats relative to sham-intubated controls. Specifically, the learning-related changes in expression observed in the prefrontal cortex will be absent in FASD rats, which fail to learn the CPFE, relative to normal rats who acquire the CPFE.

8.1.1 Methods and Materials

8.1.1.1 Subjects

Animal husbandry was as described previously (Chapter 6).

8.1.1.2 Alcohol Dosing and BAC

As described previously (Chapter 6: *Alcohol Dosing*) animals were randomly assigned to either receive a single-binge dose of alcohol from PD 7 through PD 9 (5.25 g/kg/day; Group EtOH) or sham intubation (Group SI). Blood alcohol concentration (BAC; Table 8.1) was determined using blood samples drawn from tail-snips 2 hr after PD 7 alcohol dosing.

8.1.1.3 Apparatus, Stimuli, and Procedure

The apparatus, stimuli and behavioral procedures were as described in Chapter 6: *Behavioral Procedures.* The current experiment utilized the single-exposure CPFE protocol and tissue was collected 30 (±3) min after immediate foot-shock on the training day.

8.1.1.4 *In situ* hybridization

Tissue collection, *in situ* hybridization and image analyses were as describe in Chapter 6: *In situ hybridization*.

8.1.2 Results of Experiment 8.1: Impaired CPFE in FASD Rats Associated with Decreased mRNA Expression in Prefrontal Sub-regions

8.1.2.1 Body Weights and BACs (Table 8.1)

Body weight averages for the current experiment appear in Table 8.1. All groups gained a significant amount of weight over the dosing period (PD 7–9) and up

to the age of testing (PD 31). However, a transient growth impairment was evident on PD 9 in Group EtOH rats. A 2 (Treatment: SI v EtOH) x 3 (Days: PD 7, 8, 9) repeated measures ANOVA on PD 7 and PD 9 body weights revealed no main effect of Treatment [$F(1, 95) = 2.56$, $p = .11$], however there was a main effect of Days [$F(1, 95) = 458.40$, $p < .001$], and a Treatment x Days interaction [$F(1, 95) = 44.97$, $p < 0.001$]. Newman-Keuls post hoc test showed that although body weights did not differ between treatment groups at PD 7 (p's > 0.36), at PD 9, Group EtOH body weights were significantly lower than Group SI ($p < 0.001$).

A 2 (Treatment: SI v EtOH) x 2 (Sex: Male v Female) factorial ANOVA on PD 31 body weights (combined across all sampling and behavioral conditions) revealed a significant main effect of Sex [$F(1, 93) = 41.32$, $p < .001$], with males weighing more than females. No main effect of Treatment or Sex x Treatment interaction was found (F's > .51), indicating a lack of growth effects of alcohol at the time of testing. BACs were obtained from blood samples taken on PD 7 from alcohol-exposed rats (Table 8.1). BACs are similar to those we have previously reported at this alcohol dose (e.g. Jablonski & Stanton, 2014; Murawski & Stanton, 2011).

	Body Weight (g)				BAC (mg/dL)
Treatment	PD7	PD9	PD31-32 (males)	PD31-32 (females)	PD7
SI	16.52 ± 0.24	20.78 ± 0.34	106.27 ± 1.99	94.67 ± 1.24	n/a
EtOH	16.90 ± 0.26	17.27 ± 0.25	104.50 ± 2.05	94.17 ± 1.27	417.5 ± 14.54

Table 8.1 Average body weights (grams, ± SE) for animals sacrificed following training or retained for CPFE behavior are given from the two treatment groups (SI, sham intubated; EtOH, 5.25 g/kg/d over PD 7–9) during the first (PD 7) and last (PD 9) day of dosing. Weights were taken on the first day of behavioral training (PD 30-31, Preexposure day) from all groups. Average blood alcohol concentration (BAC) obtained from blood samples collected on PD 7 from alcohol-exposed rats are given in mg/dL. Group EtOH body weights were significantly less than Group SI only at PD9.

8.1.2.2 Behavioral Results (Figure 8.1)

After outlier exclusion ($n_{\text{SI Pre}} = 1$, $n_{\text{EtOH Pre}} = 1$, $n_{\text{Pooled Alt Pre}} = 1$) behavioral analyses were completed on the remaining 34 rats [$n_{\text{SI Pre}} = 11$ ($n_{\text{male}} = 7$, $n_{\text{female}} = 4$), $n_{\text{EtOH Pre}} = 10$ ($n_{\text{male}} = 5$ $n_{\text{female}} = 5$) $n_{\text{SI Pooled Alt Pre}} = 13$ ($n_{\text{male}} = 6$, $n_{\text{female}} = 7$)]. There were no differences in freezing scores between SI and EtOH Alt-Pre animals [$t(11) = -0.81$, $p < .44$] so this group was collapsed into a pooled Alt-Pre condition. A 2 (Sex: Male v Female) x 3 (Condition: pooled Alt Pre, SI Pre and EtOH Pre) ANOVA revealed no main [$F(1, 28) = 0.65$, $p > .42$] or interaction [$F(2, 28) = 0.12$, $p > .88$] effects of sex so all groups are collapsed across this variable (Figure 8.1). One-way ANOVA revealed a significant main effect of group [$F(2, 31) = 12.86$, $p < .001$]. SI Pre freezing was significantly higher than both EtOH Pre ($p < .001$) and pooled Alt-Pre ($p < .001$) which did not differ ($p > .61$). Neonatal alcohol exposure completely abolished the CPFE in adolescent rats.

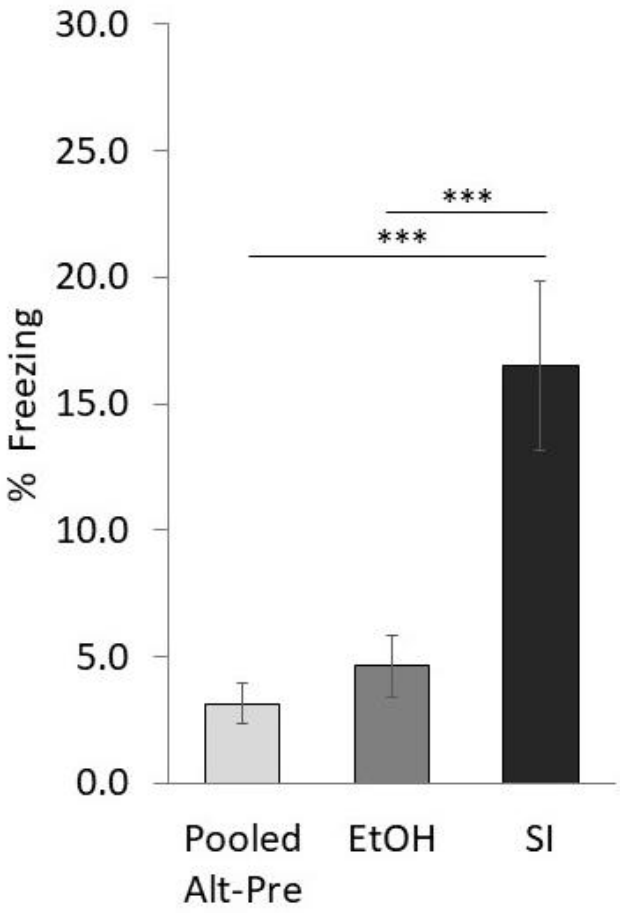

Figure 8.1 Mean (±SEM) percent freezing during the 5 min testing phase. Group SI froze significantly more than Group EtOH and Group pooled Alt-Pre, which did not differ, indicating a significant CPFE in group SI but not EtOH. ***$p < .001$.

8.1.2.3 CPFE Post-Training Egr-1 mRNA Expression

Twelve animals in the HC (n_{SI} = 6, n_{EtOH} = 6) and 48 animals from the training sampling conditions ($n_{SI\ Pre}$ = 12, $n_{SI\ Alt\text{-}Pre}$ = 12, n_{EtOH} = 12, $n_{EtOH\ Alt\text{-}Pre}$ = 12) were assayed. One subject from the PL and 6 subjects from the CA1/LA were removed from analyses due to tissue damage or labeling issues. After outlier exclusion (0-2 animals per group), CA1 [n_{HC} = 1, $n_{SI\ Pre}$ = 1, $n_{SI\ Alt\text{-}Pre}$ = 2, $n_{EtOH\ Pre}$ = 1, $n_{EtOH\ Alt\text{-}Pre}$ = 0], LA [n_{HC} = 0, $n_{SI\ Pre}$ = 1, $n_{SI\ Alt\text{-}Pre}$ = 1, $n_{EtOH\ Pre}$ = 1, $n_{EtOH\ Alt\text{-}Pre}$ = 1], PL [n_{HC} = 1, $n_{SI\ Pre}$ = 2, $n_{SI\ Alt\text{-}Pre}$ = 1, $n_{EtOH\ Pre}$ = 1, $n_{EtOH\ Alt\text{-}Pre}$ = 1], and IL [n_{HC} = 2, $n_{SI\ Pre}$ = 2, $n_{SI\ Alt\text{-}Pre}$ = 1, $n_{EtOH\ Pre}$ = 2, $n_{EtOH\ Alt\text{-}Pre}$ = 1], the final number of subjects in each group were CA1 [n_{HC} = 9 (n_{SI} = 5; n_{EtOH} n = 4), $n_{SI\ Pre}$ = 10, $n_{SI\ Alt\text{-}Pre}$ = 10, $n_{EtOH\ Pre}$ = 10, $n_{EtOH\ Alt\text{-}Pre}$ = 10], LA [n_{HC} = 10 (n_{SI} = 6; n_{EtOH} n = 4), $n_{SI\ Pre}$ = 10, $n_{SI\ Alt\text{-}Pre}$ = 11, $n_{EtOH\ Pre}$ = 9, $n_{EtOH\ Alt\text{-}Pre}$ = 9], PL [n_{HC} = 11 (n_{SI} = 6; n_{EtOH} n = 5), $n_{SI\ Pre}$ = 9, $n_{SI\ Alt\text{-}Pre}$ = 11, $n_{EtOH\ Pre}$ = 11, $n_{EtOH\ Alt\text{-}Pre}$ = 11, IL [n_{HC} = 12 (n_{SI} = 6; n_{EtOH} n = 6), $n_{SI\ Pre}$ = 10, $n_{SI\ Alt\text{-}Pre}$ = 11, $n_{EtOH\ Pre}$ = 10, $n_{EtOH\ Alt\text{-}Pre}$ = 11].

Training day *Egr-1* expression was elevated in the PL, IL and CA1 of the experimental groups compared to HC controls but not in the LA. (Figure 8.2). One-way ANOVA revealed no main effect of Condition [$F(4, 44) = 2.10$, $p < .098$) in LA. There was a main effect of Condition [$F(4, 44) = 3.66$, $p < .02$) in CA1, however Dunnett's test showed that only group EtOH Alt-Pre significantly differed from HC ($p < .01$) in CA1 (Figure 8.2).

There was a main effect of Condition in both the PL [$F(4, 48) = 13.16$, $p < .001$) and IL [$F(4, 47) = 18.06$, $p < .001$). Dunnett's test showed that all experimental groups in the PL (p's < 0.05) and IL (p's < 0.02) were higher than HC. For both regions, Newman-Keuls showed that gene expression in Group SI Pre was higher than all other experimental groups (p's < 0.02) however no difference in expression (p's >

.05) was observed between groups SI Alt-Pre, EtOH Pre and EtOH Alt-Pre in either region.

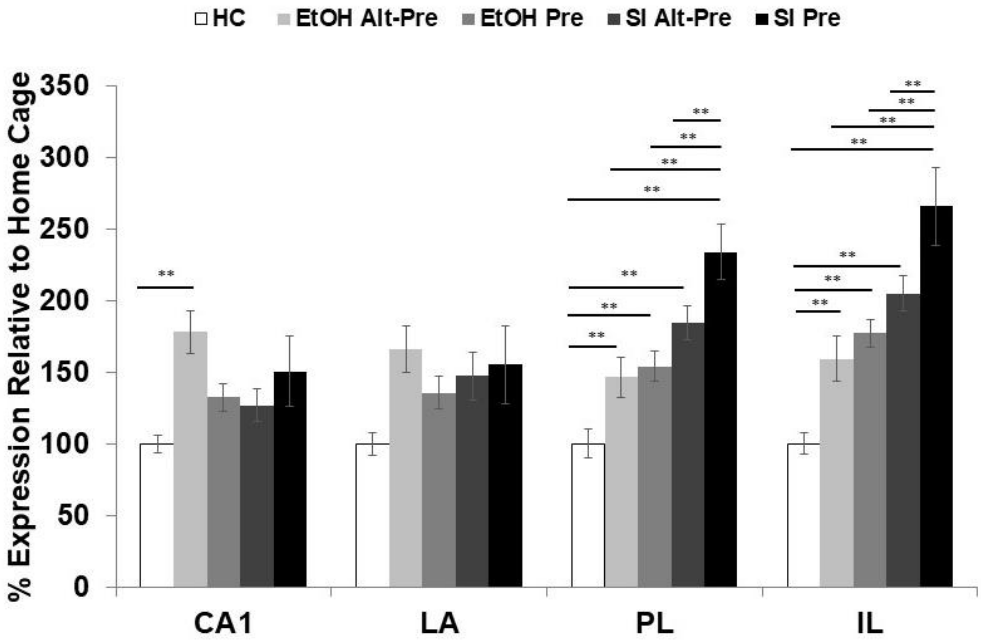

Figure 8.2 Mean (±SEM) percent expression of *Egr-1* mRNA compared to HC following immediate shock training. Group SI Pre displayed significantly higher *Egr-1* mRNA than all other groups in the PL and IL. In the PL and IL, groups SI Alt-Pre, EtOH Pre and EtOH Alt-Pre did not differ significantly in their relative expression although all groups were significantly elevated above HC. There were no group differences in the LA or CA1 except that the group EtOH Alt-Pre was significantly elevated above HC. * $p < .05$, ** $p < .01$.

8.1.3 Summary of Findings

The current experiment examined the effect of neonatal alcohol exposure on *Egr-1* mRNA expression during the CPFE. Similar to that observed in normal rats (Asok et al., 2013; Chakraborty et al., 2016; Schreiber et al., 2014), group SI Pre showed a learning-related increase in *Egr-1* expression following immediate foot-shock training relative to non-associative controls (i.e. SI Alt Pre). In contrast, the learning related increase above EtOH Alt Pre was not observed, although animals exposed to alcohol (EtOH Pre and Alt Pre) did show increased expression relative to HC,. These results illustrate impaired learning-related prefrontal *Egr-1* expression that is associated with impaired contextual learning in alcohol exposed rats.

8.1.4 Discussion

The current experiment examined the impact of neonatal alcohol on 1) contextual fear learning in the CPFE and 2) experience-induced *Egr-1* mRNA expression following context-shock association in adolescent (PD 31) rats. Following a high binge dose of alcohol (5.25 g/kg/day) administered from PD 7-9, contextual conditioning using the CPFE was disrupted in adolescent rats replicating previous reports from our lab (Jablonski & Stanton, 2014; Murawski & Stanton, 2011). Neonatal alcohol exposure also disrupted prefrontal *Egr-1* mRNA expression during the CPFE. Following training, Group EtOH Pre showed reduced mPFC *Egr-1* expression relative to Group SI Pre such that there was no difference in expression between the Group EtOH Pre and non-associative control groups (Group SI Alt-Pre and EtOH Alt-Pre). This effect was not observed in the amygdala or hippocampus. Thus, alcohol-induced reductions in learning-related mPFC plasticity may contribute to CPFE deficits observed in adolescent rats neonatally exposed to alcohol.

Perinatal alcohol exposure produces persistent region-specific immediate early gene (IEG) reductions across several models of FASDs (Chapter 4). Frontal levels of *Arc* and *c-fos*, which increase in response to social experience in control rats, show impaired induction in alcohol-exposed rats (D. A. Hamilton et al., 2010a; D. A. Hamilton et al., 2010b). Importantly, following a T-maze spatial alternation task, Nagahara and Handa (1995) showed a significant impairment in performance for alcohol-exposed animals with a 60 s delay between alternation responses. Relative to non-exposed controls, alcohol-exposed animals showed reduced *c-fos* and *jun B* mRNA expression in the prefrontal cortex but not the hippocampus. Collectively, these results illustrate that perinatal alcohol exposure disrupts experience-dependent induction of prefrontal IEGs.

NMDA receptor-dependent neuronal plasticity is integral for proper learning and memory (F. Li & Tsien, 2009; Luscher & Malenka, 2012; Malenka & Nicoll, 1993; Shimizu, Tang, Rampon, & Tsien, 2000; Tsien, Huerta, & Tonegawa, 1996) and the effects of alcohol-exposure on IEG expression is likely the result of disrupted function in molecular systems responsible for neuronal plasticity that begins during development (Fontaine, Patten, Sickmann, Helfer, & Christie, 2016; Medina, 2011). During a learning event, activation of NMDA receptors in normal animals triggers a molecular cascade that results in the induction of plasticity-related genes like *c-fos*, *arc* and *Egr-1* (Alberini, 2009; Medina, 2011; Orsini & Maren, 2012; Veyrac et al., 2014). Prenatal alcohol exposure alters hippocampal NMDA receptor subunit composition leading to disrupted NMDA-receptor dependent LTP and disrupted IEG activity (Brady et al., 2013; Medina, 2011). Importantly, intracerebroventricular (i.c.v.) antagonism of NMDA receptors during conditioning abolishes retention of

contextual fear learning and the associated increase in *Egr-1* mRNA expression in the amygdala (Malkani & Rosen, 2001). Thus, alcohol-related disruptions in neuronal plasticity and subsequent cognitive processing is at least in part the result of disrupted NMDA-dependent plasticity that begins in development and persists throughout development. Although these alcohol-related plasticity effects have been studied extensively in the hippocampus, more work is needed to examine similar plasticity effects in the prefrontal cortex.

The mPFC plays a role in learning the context on the preexposure day and in context-shock association or consolidation on the training day (Chapter 2). Indeed, mPFC infusions of muscimol (Heroux et al., 2017) or scopolamine (Robinson-Drummer et al., 2017) on either day disrupts the CPFE (i.e retention-test freezing). Neonatal alcohol exposure causes structural damage in the mPFC like alterations in spine density, distribution and morphology (D. A. Hamilton et al., 2010a; Lawrence et al., 2012; Whitcher & Klintsova, 2008) as well as reductions in basilar dendritic length and branching (Granato et al., 1995; D. A. Hamilton et al., 2010a) that could contribute to learning deficits during either phase of conditioning. However, recent work from our lab examining the effect of PD 7-9 alcohol exposure on training-day post-shock freezing suggests that training day memory processes (e.g., consolidation of the context-shock association), rather than preexposure day processes (e.g., forming and consolidating a contextual representation) are responsible for alcohol-related impairments in the CPFE (Jablonski & Stanton, 2014). Thus, differential training day *Egr-1* expression in the mPFC likely reflects an inability of alcohol-exposed animals to consolidate the context-shock association acquired following foot-shock training.

The current results suggest that neonatal alcohol exposure may disrupt prefrontal neural circuitry necessary for long-term retention of context-specific fear memories following foot-shock training. Whether these prefrontal disruptions result in impaired consolidation or retrieval of the context-shock association remains to be determined. Targeting specific components of prefrontal dysfunction may be beneficial for reversing deficits in synaptic plasticity and improving behavioral outcomes, providing a potential mechanism of intervention for FASDs.

Chapter 9

ANTAGONISM OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN MEDIAL PREFRONTAL CORTEX DISRUPTS THE CONTEXT PREEXPOSURE FACILITATION EFFECT

The previous chapters have demonstrated the ontogeny of *Egr-1* expression in normally developing rats as well as the detrimental effects of neonatal alcohol exposure not only on fear learning but on normal molecular function in the prefrontal cortex. As noted previously, neonatal alcohol exposure disrupts acquisition of the CPFE (Chapter 4 and 9; G. F. Hamilton et al., 2011; Jablonski & Stanton, 2014; Murawski & Stanton, 2010, 2011) and this behavioral impairment can be rescued by enhancing cholinergic function during behavioral testing (Dokovna et al., 2013). Other labs have also shown that alcohol-induced behavioral and cognitive impairments as well as molecular changes in the hippocampus and prefrontal cortex can be rescued with choline supplements (Monk et al., 2012; Otero et al., 2012; Schneider & Thomas, 2016; Thomas et al., 2007; Thomas et al., 2010; Thomas & Tran, 2012; Wagner & Hunt, 2006). Taken together, these studies support a significant role of cholinergic dysfunction in learning and memory impairments observed in FASD. The mPFC receives a large cholinergic input (Everitt & Robbins, 1997; Henny & Jones, 2008; Raghanti et al., 2008) and is necessary for behavioral conditioning during the CPFE (Heroux et al., 2017). This raises the possibility that mPFC cholinergic dysfunction may contribute to learning impairments observed following neonatal alcohol exposure. However, it is not known whether the CPFE depends on cholinergic receptor function in mPFC. The experiments in the present chapter examined this question (Robinson-Drummer et al., 2017).

9.1 Experiment 9.1: mPFC Muscarinic Cholinergic Function is Necessary for Successful Performance of the CPFE (Figure 9.1)

The current experiment describes the effect of infusions of a scopolamine, cholinergic antagonist, prior to all phases of the CPFE on conditioned freezing during the final test phase. The experiment that follows (9.2) examined behavioral effects of these infusions performed separately prior to each experimental phase.

9.1.1 Materials and Methods

9.1.1.1 Subjects

Animal husbandry was as described previously (see Chapter 6).

9.1.1.2 Surgery

On PD 29, juvenile rats were taken from post-weaning group housing and anesthetized with an intraperitoneal ketamine/xylazine injection and subcutaneous buprenorphine near the incision site to reduce post-operative discomfort. A fused double-guide cannula (Plastics One, Roanoke, VA) was implanted bilaterally to terminate above the prelimbic region of medial prefrontal cortex using the following coordinates: anteroposterior (AP) +9.0 mm and mediolateral (ML) ± 0.6 mm relative to interaural midline and dorsoventral (DV) -2.3 mm relative to the top of the skull. Cannula were fixed in placed using dental acrylic and curved "skull hooks" (Robinson-Drummer et al., 2016; Schiffino et al., 2011; Watson & Stanton, 2009). Following surgery, dummy internals and dust caps were inserted in the guide cannula to reduce occlusion and rats were allowed to recover in individual cages with electric heating pads placed under half of the cage floor. Animals were allowed to recover for approximately 24 hr until their cannula were cleared with phosphate buffered saline

vehicle (PBS; Fisher Scientific, Waltham, MA) the following day. For each animal, 0.25 μL of the PBS was infused per side to ensure that no cannulas were occluded.

9.1.1.3 Drug Infusion

Depending on their drug condition (see *Apparatus, Stimuli and Procedure* below) rats received microinjections of either PBS or scopolamine hydrobromide (Scop; Sigma Aldrich, St. Louis, MO) dissolved in PBS approximately 10 min before behavioral procedures. Animals were hand held while scopolamine (140 μg/μL dissolved in PBS) was infused at a rate of 0.25 μL per minute for a single minute, administering 35 μg of scopolamine per side per animal. This dose has been used previously in our lab (Brito, Davis, Stopp, & Stanton, 1983; Robinson-Drummer et al., 2016) and similar doses of scopolamine have been infused intra-cranially in other labs (Chang & Liang, 2012; Gale et al., 2001; Rogers & Kesner, 2004). Drug injectors were left in place for an additional minute to allow diffusion of drug before removal. PBS control animals were administered the same volume of PBS at the same rate as scopolamine animals. A 0.25 μL infusion diffuses about 1 mm from the cannula tip ensuring that the spread of the drug is restricted to the prefrontal cortex. This is based on our other studies using injected dyes (Jablonski, Watson, & Stanton, 2010) or labelled muscimol (Heroux et al., 2017). After infusions, animal were returned to their home cage until conditioning.

9.1.1.4 Apparatus, Stimuli and Procedure

The current study utilized materials and methods as previously described in Chapter 6 with the following additions. The current study utilized the multiple-exposure protocol: animals received a 5 min exposure followed by five 1 min

exposures separated by 1 min. On the training day, *two* 1.5 mA, 2 s foot-shocks presented 1 s apart were used instead of a single shock in order to overcome a slight depression in behavioral conditioning associated with cannulation surgery.

For all studies, animals were randomly assigned to receive either PBS or scopolamine (Scop) prior to behavioral conditioning. Group Pre animals were split between drug (PBS or Scop) and infusion day while the Alt-Pre group was pooled across drug. Group Alt-Pre has historically performed similarly regardless of drug treatment (Dokovna et al., 2013; Heroux et al., 2017; Jablonski et al., 2012; Robinson-Drummer et al., 2016; Schiffino et al., 2011) and pooling reduces the number of animals needed for completion of the study. Sex was counterbalanced within litters for each preexposure, drug and infusion day group and was collapsed within an experimental group.

9.1.1.5 Statistical Analysis

Data and statistical analyses were as described previously (Murawski & Stanton, 2010; Robinson-Drummer et al., 2016). Planned comparisons and post-hoc Newman-Keuls tests were used to assess any significant effects revealed by ANOVA.

9.1.2 Results of Experiment 9.1 (Figure 9.1)

Thirty-five Long-Evans rats from 22 litters were assigned to groups by drug (PBS v Scop) and preexposure condition (Pre v Alt-Pre) with testing day freezing being statistically compared across groups. There was no effect of drug observed in Group Alt-Pre [$F(1,9) = 2.10$, $p = 0.18$] so this variable was pooled across drug. Four subjects were removed due to misplaced cannula (Pre PBS $n = 1$, Pre Scop $n = 3$; Figure 9.1, left panel). A 2 (Sex: Male, Female) x 3 (Condition: pooled Alt-Pre, Pre

PBS, Pre Scop) factorial ANOVA revealed no significant main effect of Sex [$F(1, 22) = 1.42$, $p = .25$] or Sex by Condition interaction [$F(2, 22) = 1.92$, $p = .32$] so all analyses were collapsed across this variable. Subsequent analyses are the result of a three group one-way ANOVA (pooled Alt-Pre, Pre Scop, and Pre PBS; Figure 9.1, right panel). A single outlier was removed from each of the three experimental groups and final group sizes were as follows: pooled Alt-Pre $n = 11$, Pre Scop $n = 8$, and Pre PBS $n = 9$. ANOVA revealed a significant effect of condition [$F(1, 25) = 22.26$, $p < .001$] such that freezing in the Pre PBS group was significantly elevated above both Pre Scop and pooled Alt-Pre (p's $< .001$) however there was no difference between groups Pre Scop and Alt-Pre ($p = .75$), indicating that the drug abolished the CPFE. These results suggest a significant role for mPFC cholinergic function in successful performance of the CPFE but they do not indicate whether a particular phase of the CPFE is critical for this effect.

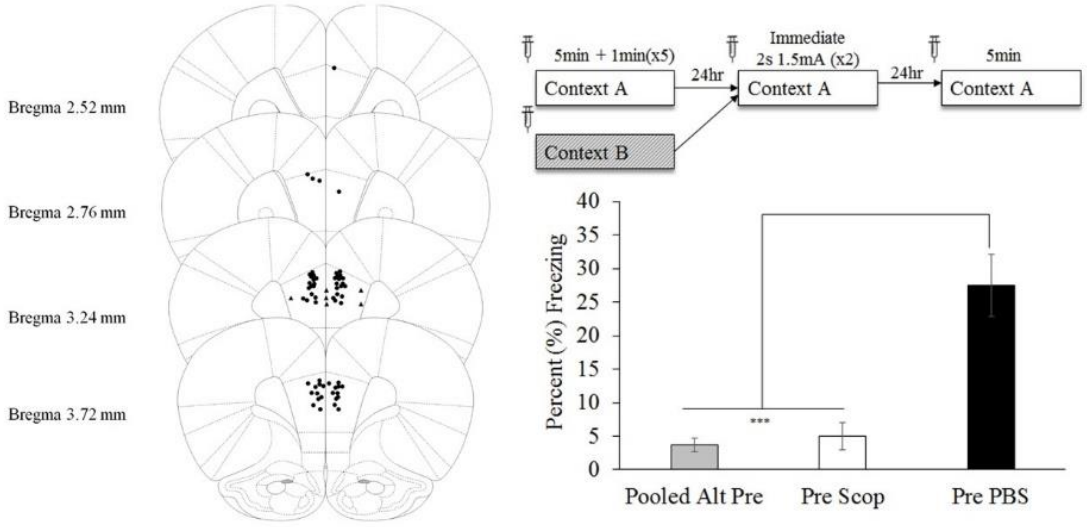

Figure 9.1 (left) Schematic representation of injection cannula tip placement in the mPFC for Experiment 9.1. Animals included in final analyses are represented by filled black circle while animals excluded as mPFC placement misses are filled black triangle. Extremely anterior (Bregma 4.68 mm or more) or posterior (Bregma 2.28mm or less) were automatically excluded and are not represented in the figure. Coronal brain images are adapted from the rat brain atlas of (Paxinos & Watson, 2013). (right,top) Schematic of CPFE and drug infusion procedures and (right, bottom) Mean (±SEM) percent time freezing during a 5 min test of conditioned fear in Experiment 9.1. Animals were given either PBS or scopolamine prior to all three conditioning phases of the CPFE. Comparisons reflect a one-way ANOVA for group (pooled Alt-Pre, Pre Scop and Pre PBS). Scopolamine significantly impaired CPFE performance on the testing day. Asterisks indicate significance relative to Pre PBS group. *** = $p < .001$.

9.2 Experiment 9.2: Effects of mPFC Scopolamine Infusions Performed Separately Before Each Phase of the CPFE (Figure 9.2)

The previous results (Experiment 9.1) indicate a significant contribution of mPFC cholinergic function to contextual fear conditioning during the CPFE. In order to determine which psychological processes are disrupted by prefrontal cholinergic antagonism, the following experiment targeted context learning (Experiment 9.2a), context-shock learning (Experiment 9.2b) or fear memory retrieval (Experiment 9.2c) by administering scopolamine prior to only a single experimental phase.

9.2.1 Materials and Methods

All materials and methods were identical to that described in Experiment 9.1 except that drug infusions were administered prior to only the preexposure (Experiment 9.2a), training (Experiment 9.2b) or testing day (Experiment 9.2c) of the CPFE procedure. Cannula placements were similar to Experiment 9.1 but representative figures for Experiments 9.2a-c are provided in the Appendix (Figures A1-A3).

9.2.2 Results of Experiment 9.2a: Scopolamine Infusions into the mPFC Prior to the Preexposure Day Impairs Contextual Learning During the CPFE (Figure 9.2a)

Forty-one rats from 26 litters were grouped by drug (PBS v Scop) and preexposure condition (Pre v Alt-Pre). Four animals were removed from analyses due to misplaced cannula (Alt-Pre Scop $n = 1$, Pre PBS $n = 2$, Pre Scop $n = 1$). Again, lack of Sex [$F(1, 35) = 0.67$, $p = .42$] or Sex x Condition interaction [$F(2, 35) = 2.32$, $p = .11$] effects led us to pool subsequent analyses across this factor. There was a significant effect of drug in the Alt-Pre group [$F(1,11) = 30.60$, $p < .001$] so pooling across drug was not possible in this experiment. A single outlier was removed from

groups Alt-Pre PBS, Alt-Pre Scop and Pre Scop and two outliers were removed from group Pre PBS. Final group sizes were as follows: Alt-Pre PBS n = 7, Alt-Pre Scop n = 6, Pre PBS n = 16, Pre Scop n = 1). ANOVA revealed a significant effect of preexposure condition [$F(3, 37) = 13.21$ $p < .001$; Figure 9.2a]. Planned comparisons revealed a significant CPFE as measured by an increase in freezing in Pre PBS relative to Alt-Pre PBS [$F(1, 37) = 5.05$, $p = .03$] and Alt-Pre Scop [$F(1, 37) = 20.74$, $p < .001$]. In addition, Pre PBS freezing was significantly elevated above Pre Scop [$F(1, 37) = 31.70$, $p < .001$] whereas there was no difference in freezing observed between Alt-Pre Scop and Pre Scop [$F(1, 37) = 0.003$, $p = .95$]. Thus the CPFE was eliminated following mAChr antagonism prior to context preexposure. These results indicate that muscarinic-type cholinergic function is necessary for context learning (or possibly consolidation of this learning) on the preexposure day of the CPFE.

9.2.3 Results of Experiment 9.2b: Training Day Processes are Significantly Impaired by mPFC Muscarinic Antagonism During the CPFE (Figure 9.2b)

To examine prefrontal cholinergic involvement in training-day processes, scopolamine was infused bilaterally into the mPFC prior to immediate foot-shock (two 2 s, 1.5 mA) training. Forty Long-Evans rats from 26 litters were grouped by drug (PBS v Scop) and preexposure condition (Pre v Alt-Pre). Three animals were removed from analyses due to misplaced cannula (Alt-Pre Scop n = 1, Pre PBS n = 1, Pre Scop n = 1). There was no effect of drug in the Alt-Pre groups [$F(1, 11) = 1.32$, $p = .27$] so a pooled Alt-Pre group was used. Data were also pooled across sex because the 2 (Sex: Male, Female) x 3 (Condition: pooled Alt-Pre, Pre PBS, Pre Scop) ANOVA revealed no main effect [$F(1, 29) = .27$, $p = .61$] or interaction [$F(2, 29) = 0.08$, $p = .93$] involving this factor. A single outlier was removed from each of the three

experimental groups and final group sizes were as follows: pooled Alt-Pre n = 13, Pre Scop n = 12, and Pre PBS n = 10. A one-way ANOVA (pooled Alt-Pre, Pre Scop, and Pre PBS) revealed a significant effect of condition [$F(1, 32) = 18.27$, $p < .001$] such that Pre PBS was significantly elevated above both Pre Scop and pooled Alt-Pre (p's < .001). However, there was no difference between Pre Scop and pooled Alt-Pre (p = .54). These results demonstrate a necessity of mPFC muscarinic activity for training-day processes during the CPFE.

9.2.4 Results of Experiment 9.2c: Intra-mPFC Scopolamine Administered Prior to Testing Impairs Fear Memory Retrieval or Performance of the CPFE in Females But not Male Rats (Figure 9.2c)

The current experiment infused scopolamine into the mPFC prior to fear memory testing to examine the role of mAChr function on memory retrieval or expression. Forty three rats from 21 litters were grouped by drug (PBS v Scop) and preexposure condition (Pre v Alt-Pre). Six animals were removed due to misplaced cannula (Alt-Pre PBS n = 2, Pre PBS n = 1, Pre Scop n = 3). Alt-Pre groups were unaffected by drug [$F(1, 10) = .004$, $p = .95$] so a pooled Alt-Pre group was used. Although 2 (Sex: Male, Female) x 3 (Condition: pooled Alt-Pre, Pre PBS, Pre Scop) ANOVA revealed no main effect of Sex [$F(1, 34) = .12$, $p = .73$], there was an effect of Condition [$F(1, 34) = 14.60$, $p < .001$] and a Sex x Condition interaction [$F(2, 34) = 5.40$, $p < .01$]. The interaction was driven by a significant difference between female and male animals in the Pre Scop group (p = .01) so subsequent analyses were not pooled across this variable. One outlier was removed from each of the six groups. Group sizes were as follows: Female pooled Alt-Pre n = 5, Female Pre PBS n = 5, Female-Pre Scop n = 6, Male pooled Alt-Pre n = 7, Male Pre PBS n= 8, Male-Pre Scop n = 9. Newman-Keuls post hoc tests revealed that in males, there was no

significant difference observed between Pre Scop and Pre PBS (p = .94) and a significant CPFE was evident regardless of testing day drug (ps < .05 relative to pooled Alt-Pre; Figure 9.2c, right panel). However, in females the significant CPFE observed between Pre PBS and pooled Alt-Pre (p < .001) was not evident in Pre Scop vs. pooled Alt-Pre (p = .81; Figure 9.2c, left panel). This evidence that mPFC scopolamine influences expression of the CPFE in females but not males should be regarded with caution as it may reflect sampling error. Analysis of effect sizes indicates that twice as much of the variance results from the drug condition (η_p^2 = .462) than the sex x drug condition interaction (η_p^2 = .241) and nearly none of the variance is due to sex alone (η_p^2 = .003). We have also not seen similar sex differences following systemic or intra-hippocampal scopolamine administration (Robinson-Drummer et al., 2016). On the other hand, there is some evidence that the mPFC may contribute to sex differences observed in eyeblink conditioning following stress (Maeng & Shors, 2013; Maeng, Waddell, & Shors, 2010; Wood & Shors, 1998). Whether the present findings represent this type of outcome or are merely sampling error is a question that requires further study.

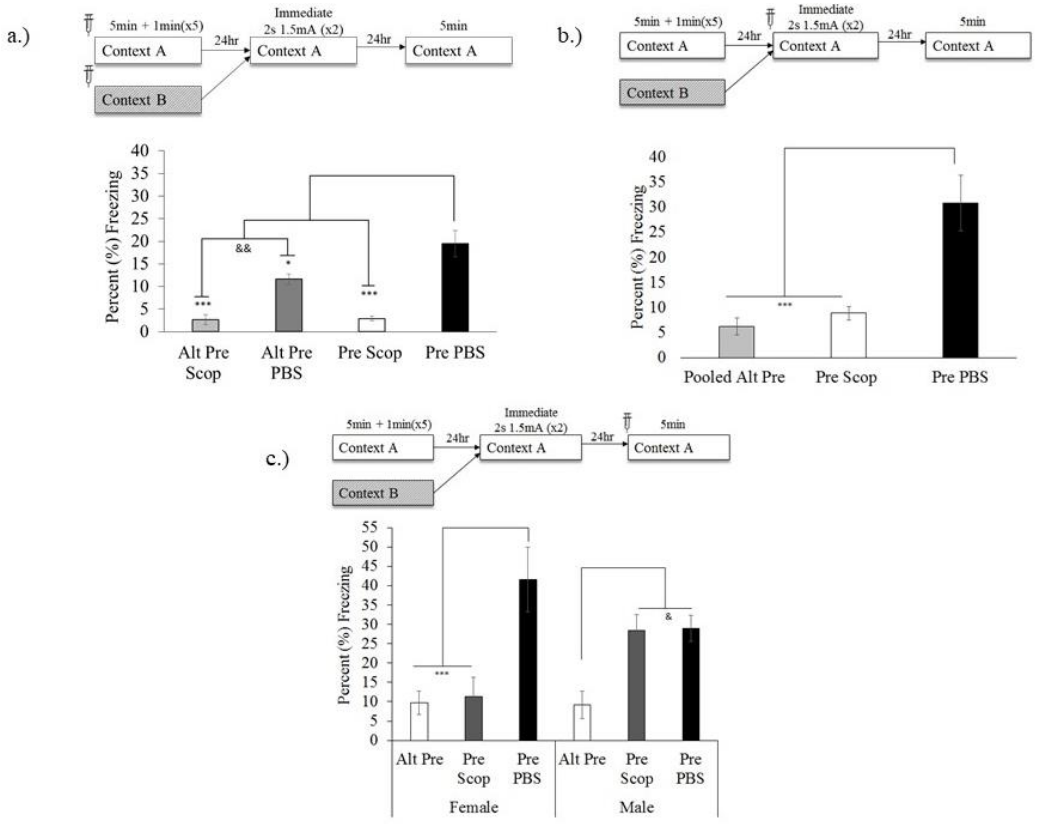

Figure 9.2 Mean (±SEM) percent time freezing during a 5 min test of conditioned fear following pre-conditioning drug infusion in Experiment 9.2 (a-c). Scopolamine administered prior to context preexposure (a) or immediate foot-shock training (b) significantly impaired CPFE performance on the testing day. Testing day scopolamine infusions impaired the CPFE in female but not male rats (c). Asterisks indicate significance relative to Pre PBS group. Ampersand indicates significance within exposure condition. & = $p < .05$; && = $p < .01$; * = $p < .05$; ** = $p < .01$; *** = $p < .001$.

9.3 Summary of Findings

The previous experiments examined the role of muscarinic-type acetylcholine receptors (mAChr) in contextual fear conditioning during the CPFE. The results reveal that prefrontal mAChr are necessary for context learning on the preexposure day and context-shock association on the training day in both male and female rats. Prefrontal antagonism prior to testing, in contrast produced mixed results with males showing no indication of impairment while females were significantly impaired in the CPFE following prefrontal cholinergic antagonism.

9.4 Discussion

The current experiments demonstrate a necessary role of muscarinic cholinergic receptor function in the medial prefrontal cortex in the CPFE. This function contributes to learning-related activity on the preexposure (Experiment 9.2a) and training (Experiment 9.2b) days but not to memory retrieval or CPFE performance on the testing day (Experiment 9.2c), at least in males. The effects observed in Experiments 9.2a and 9.2b are unlikely to be state-dependent as this account predicts that there would be no effect of scopolamine when the drug is given during all three phases of the CPFE, a prediction that was not supported by the outcome of Experiment 9.1.

The results of Experiments 9.2a and 9.2b support and extend previous reports of a role of the mPFC in the acquisition of contextual fear conditioning. Acquisition of background contextual conditioning (i.e. when the context competes with a discrete CS as a predictor of the US) is significantly impaired by disrupting neural activity or plasticity in the mPFC prior to conditioning (Gilmartin & Helmstetter, 2010; Gilmartin et al., 2013). However, the co-occurrence of context learning and context-

shock association during sCFC in these previous studies makes it difficult to parse out which process is disrupted by mPFC scopolamine. The current results extend previous knowledge by revealing a necessary role for cholinergic mPFC function for acquisition of both the context representation and the context-shock association. Intra-hippocampal scopolamine significantly impairs CPFE performance when infused post-training while post-preexposure infusions have no effect suggesting cholinergic antagonism creates a consolidation deficit on the training day and an encoding deficit on the preexposure day (Chang & Liang, 2012). Other results from our lab (Heroux et al., 2017) support a failure in consolidation (or reconsolidation) as the mechanism by which training day scopolamine impairs CPFE performance. Prefrontal inactivation spares post-shock freezing indicating that mPFC inactivation does not prevent rats from retrieving the context representation and momentarily associating it with a foot-shock. Furthermore, if scopolamine targets consolidation processes, post-conditioning infusions should have the same effect on CPFE performance as the current experiments. Although this has been reported following intra-hippocampal scopolamine (Chang & Liang, 2012), additional experimentation is necessary to dissociate the effect of mPFC scopolamine on acquisition versus consolidation of contextual fear conditioning.

The lack of a deficit following pre-testing scopolamine in Experiment 9.2c was surprising as the mPFC (specifically the prelimbic region) is thought to be the primary region responsible for fear expression (Giustino & Maren, 2015). Whereas the current results suggest no contribution of the mPFC cholinergic system to testing-day processes, previous results do demonstrate a need for a functional mPFC either for memory retrieval or expression of contextual fear (Corcoran & Quirk, 2007; Heroux et

al., 2017). In their review, Giustino and Maren (2015) suggest that the mPFC is differentially recruited for fear memory acquisition or expression depending on the task requirements. However they do not speculate on the mechanism by which this task-dependent recruitment is achieved. It is likely that this recruitment is dependent on circuit-level interactions of the mPFC with other regions necessary for contextual conditioning. Prefrontal inhibition during contextual fear conditioning disrupts entorhinal-hippocampal activity and these circuit-level changes are associated with reduced fear memory during a 24 hr test (Bero et al., 2014). Additionally, in a model of mPFC regulation of attention, the ability for the mPFC to switch cue-processing modes depends on both tonic and transient cholinergic function from the basal forebrain (BF; Hasselmo & Sarter, 2011). Tonic BF cholinergic activity regulates cue-evoked glutamatergic input from thalamic nuclei that in turn may modulate transient cholinergic changes from projections that enhance cue detection. If testing day context exposure during the CPFE triggers (contextual) cue-related mAChr-type mPFC activity then testing day scopolamine would significantly impair fear memory retrieval; our results do not support this hypothesis (at least in males). Whether these types of circuit- and transmitter-level neuromodulations are acting to control mPFC involvement in the CPFE across the different phases is an interesting question to be explored in future studies.

The current findings expand upon a large body of work implicating the mPFC in acquisition of contextual fear memories (Chapter 2; Frankland & Bontempi, 2005; Giustino & Maren, 2015; Maren et al., 2013). Recently, Zelikowsky et al. (2013) showed that both the IL and PL are critically involved in contextual fear dorsal hippocampal function is compromised. Following hippocampal lesions, activity in

amygdala-projecting PL and IL cells was increased in PL cells and decreased in IL cells which the authors argue is evidence for compensatory systems reorganizing to support fear conditioning. Additionally, mPFC neuronal spiking is sensitive to re-exposure to an already acquired fear-evoking context (Baeg et al., 2001) or re-exposure to an already experienced context (Hyman et al., 2012). The neurochemical mechanisms of this activity is poorly understood and research into cholinergic contributions to context-specific mPFC function is an interesting area yet to be explored.

Based on the accumulated literature from our lab and others that use the CPFE, both the mPFC and dHPC play significant and varied roles during all three CPFE phases. This developing knowledge of contextual fear conditioning challenges previous models of system consolidation that suggest separate roles for the dHPC and mPFC in recent and remote memories, respectively (Frankland & Bontempi, 2005; Quinn, Ma, Tinsley, Koch, & Fanselow, 2008). The current report shows that the role of mPFC is not confined to remote memory. Rather, it is likely a site of early acquisition and consolidation of fear memories as well as a participant in the long term retrieval and expression of that memory (Giustino & Maren, 2015; Heroux et al., 2017). Additionally, mPFC activity (as measured by relative gene expression) during the CPFE not only changes during context exposure but increases in a learning-related way following immediate shock training (Asok et al., 2013; Schreiber et al., 2014). It seems that as new contextual conditioning parameters are explored many of the canonical models of mPFC contributions to contextual fear memory will need to be revised to include a role of the mPFC in the early acquisition, encoding and consolidation of memories.

Taken together, it appears that the mPFC contributes to the early stages of contextual fear conditioning during the CPFE (Heroux et al., 2017) and that the cholinergic system in the mPFC contributes to acquisition or consolidation of context- and context-shock learning but not retrieval or expression of this learning during the test phase of the CPFE. These findings encourage further, more nuanced exploration of mPFC involvement in the early stages of contextual fear conditioning and neurobehavioral deficits following neonatal alcohol exposure.

Chapter 10

SUMMARY AND CONCLUSIONS

The current dissertation utilized the context preexposure facilitation effect (CPFE) to explore the molecular mechanisms of learning and memory in normal and neonatally alcohol-exposed rats during development. In Chapter 3, it was noted that impairments in infant long-term memory retention largely reflect impaired memory retrieval (Josselyn & Frankland, 2012; S. Li et al., 2014; Madsen & Kim, 2016). Particularly, infant rats are fully capable of acquiring contextual fear memories however they rapidly lose the ability to retrieve either the context representation or the contextual fear memory (Jablonski et al., 2012; Rudy, 1993; Rudy & Morledge, 1994; Schiffino et al., 2011). Our report which assessed retention of context-only memory further supported this hypothesis (Robinson-Drummer & Stanton, 2015). During the CPFE, when context preexposure happened during infancy (PD 17), rats were not able to retrieve or associate the context representation with immediate foot-shock after any retention interval (1-15 day) between the two phases. Jablonski et al. (2012) previously suggested that infant rats may fail to consolidate the context representation into a retrievable (or associable) memory trace although the context representation is acquired (Rudy & Morledge, 1994). Experiments 7.1-7.3 explored this idea by examining the molecular correlate of learning (as measured by *Egr-1* expression) across ontogeny during the CPFE. Next, Experiment 8.1 examined the effects of neonatal alcohol exposure in adolescent rats on *Egr-1* expression. Lastly, Experiment 9.1-9.2 proposed a mechanism by which alcohol exposure disrupts the CPFE, i.e., by preventing learning related *Egr-1* expression of prefrontal areas in adolescent rats.

10.1 Cortical Response to Contextual Fear Learning is Altered in Infant Rodents

In Chapter 5, it was noted that immediate early genes (IEGs) like *Egr-1* are molecular components of learning that participate in the long-term consolidation of memories (Alberini, 2009, 2011; Veyrac et al., 2014). In our previous studies, *Egr-1* expression was upregulated throughout the brain following context learning and successful CPFE performance was associated with increased relative *Egr-1* expression in the prefrontal cortex of adolescent (Asok et al., 2013; Schreiber et al., 2014) and adult (Chakraborty et al., 2016) rats following the training phase of the CPFE. In conjunction with reports that suggested a significant role of *Egr-1* in contextual fear learning (J. L. Lee, 2010; Malkani & Rosen, 2001; Malkani et al., 2004) we asked *will infant amygdala, hippocampal or medial prefrontal gene expression changes differ from older animals following context preexposure and immediate shock training?* We hypothesized that PD 24 gene expression would mimic that observed in older animals however PD 17 animals would show disrupted gene expression. However using the single-exposure CPFE protocol both groups showed gene expression differences relative to older animals. Following preexposure, we were able to demonstrate a significant upregulation of *Egr-1* expression in PD 24 animals across the brain however this up regulation was absent in PD 17 rats (Experiment 7.1b). In contrast, following training, *Egr-1* levels increased similarly in PD 17 and PD 24 animals (Experiment 7.1c). These results suggest that infant rats have a fundamentally different molecular response to incidentally acquired context memory relative to older animals. Additionally, increased preexposure facilitated training day *Egr-1* expression in PD 24 animals (Experiment 7.1c) such that PD 24 rats showed similar patterns of expression as adolescent (Asok et al., 2013; Schreiber et al., 2014) and adult (Chakraborty et al., 2016) animals, however this facilitation was absent in PD 17

animals. Again these results suggest that contextual fear conditioning in PD17 rats may not utilize the same neurological circuits as that used in older animals.

At PD 24, increased context exposure is needed to achieve similar patterns of prefrontal activity during contextual fear as older animals (Experiment 7.1c-d) although the CPFE is evident regardless of *Egr-1* expression profile at this age. This suggests that *Egr-1* expression on the training day is not the mechanism of by which the CPFE develops in rats. On the other hand, preexposure day expression does track the emergence of the CPFE suggesting that *Egr-1*expression during this phase may contribute to CPFE ontogeny. Previously we suggested that impaired context representation consolidation or retrieval following the preexposure may be a mechanism of CPFE disruption in infant rats (Jablonski et al., 2012; Robinson-Drummer & Stanton, 2015). The current results contribute to the larger debate over infant context learning (Chapter 3) by suggesting a mechanism by which long-term context memory failures may happen in infant rats; impaired IEG response following preexposure may result in context memories that are poorly consolidated or retrieved.

10.2 Disrupted Molecular Activity in the mPFC Likely Subserves Alcohol-induced CPFE Deficits

In our previous reports, we concluded that impaired CPFE performance following neonatal alcohol exposure is the result of failed consolidation or testing-day retrieval of the context-shock association (Chapter 4). In order to identify a potential mechanism by which alcohol impairs the CPFE, we next asked *how does neonatal alcohol exposure change patterns of EGR-1 expression in the amygdala, hippocampus and prefrontal cortex following immediate shock training?* We hypothesized that alcohol-exposed animals would have impaired *Egr-1* expression following immediate

foot-shock training relative to non-exposed animals and this hypothesis was supported by our results. Group EtOH failed to show the CPFE, replicating our previous findings (Chapter 4), and alcohol exposure reduced cortical *Egr-1* in the mPFC (Experiment 2). Using the same dosing procedure as Experiment 8.1, Jablonski and Stanton (2014) demonstrated preserved context memory retrieval and context-shock association in EtOH rats using a post-shock test. This confirms that training-day and not preexposure day processes are disrupted in EtOH rats and suggests that the changes in prefrontal *Egr-1* expression following training reflects impaired long-term memory processes following context-shock association (even though the association itself is acquired). Interestingly, alcohol exposure did not change *Egr-1* expression in the hippocampus and amygdala although *Egr-1* expression in both areas has been implicated in acquisition of contextual fear conditioning (J. L. Lee, 2008, 2010; Malkani et al., 2004).

In previous reports that used a PD 4-9 exposure window, we concluded that the CPFE deficit we observe in alcohol exposed rats is a consequence of hippocampal structural and functional damage (G. F. Hamilton et al., 2011; Murawski et al., 2012). However the current results suggests impaired neuronal function specifically in the mPFC may underlie disruptions in contextual conditioning in PD 7-9 alcohol exposed animals. These disparate conclusions may reflect differences in teratogenic effects as a result of the various dosing procedures. Ikonomidou et al. (2000) showed that in response to ethanol exposure, peak levels of apoptosis is observed at an earlier stage for the hippocampus (about PD 3) than for the frontal cortex (about PD 7). It is possible that the PD 7-9 window (Chapter 8) and the PD 4-9 window (G. F. Hamilton et al., 2011; Murawski et al., 2012) target different components of the CPFE fear

circuit and create deficits in performance by slightly different neurological mechanisms.

Given the known role of the hippocampus in the CPFE (Chapter 1) and the recently identified role of the mPFC to context learning and fear conditioning (Chapter 2) it is possible that these regions work in tandem during the CPFE and that dysregulation in both regions contributes to the impaired performance. Thus, further experimentation which examines a larger array of IEGs across these regions (Heroux et al., 2018) has begun in order to explore the possibility of similar cooperative roles of the hippocampus and mPFC during the CPFE that has been proposed to exist for other contextual learning paradigms (Chapter 2).

10.3 Prefrontal Cholinergic Function is Necessary for Context Learning and Acquisition of Contextual Fear

Until recently the mPFC was not thought to participate in the acquisition of contextual memories (Chapter 2) however molecular activity in the mPFC is a better indicator of CPFE performance than in the hippocampus or amygdala (Experiment 2; Chapter 5). The previous results (Experiment 8.1) illustrated an association between disrupted prefrontal function and deficits in CPFE performance following alcohol exposure (Chapter 8). In order to propose a neurochemical mechanism by which alcohol exposure impairs CPFE performance, we finally asked *how does prefrontal cholinergic function contribute to contextual fear conditioning in the CPFE?* Bilateral infusions of scopolamine (35μg/side) prior to preexposure or training (i.e. immediate foot-shock) but not prior to testing significantly impaired CPFE performance (Experiment 9.2a-c). Cholinergic function in the hippocampus is critical for contextual fear conditioning (Chapter 5) however our current results also indicate a critical role of

prefrontal cholinergic function to context learning and context shock association in the CPFE. Thus, we concluded that prefrontal cholinergic dysfunction may underlie memory deficits in alcohol-exposed animals during the CPFE. This proposition is supported by numerous reports which illustrate the beneficial effects of cholinergic supplementation on dysregulated prefrontal function (Costa, Giordano, & Guizzetti, 2013; Otero et al., 2012; Schneider & Thomas, 2016; Thomas & Tran, 2012). However, additional reports from our lab and others suggest that the prefrontal cortex is not acting independently during contextual fear conditioning. During sCFC, lesioning of both the prefrontal cortex and the hippocampus impairs fear conditioning only when lesions are in contralateral hemispheres, effectively disconnecting the two regions (Zelikowsky et al., 2013). This supports a collaborative role between these regions during contextual fear conditioning (Chapter 2) that is further suggested by the observed deficits in cholinergic function for *both* the hippocampus (Robinson-Drummer et al., 2016) and the prefrontal cortex (Experiment 9.1). Cholinergic impairments may directly affect long-term learning and memory processes by interfering with normal NMDA-dependent molecular function. Muscarinic-type acetylcholine receptor (mAChr) modulation of NMDA-receptor activity and neuronal plasticity has been observed in the hippocampus (Dennis et al., 2016; Feig & Lipton, 1993; Marchi & Raiteri, 1989; M. J. Marino, Rouse, Levey, Potter, & Conn, 1998) although it is less clear whether this modulation functions similarly in the prefrontal cortex (Hasselmo & Sarter, 2011; Kurowski, Gawlak, & Szulczyk, 2015; Sarter et al., 2016). Specifically, cholinergic activity has been observed to drive NMDA-dependent plasticity processes during contextual fear conditioning (Mitsushima, Sano, & Takahashi, 2013). It is possible that reductions in cholinergic function impairs

NMDA-dependent neuronal activity and subsequently disrupts induction of plasticity molecules (i.e. immediate early gene response) necessary for long-term contextual memory. Whether the interaction between prefrontal and hippocampal cholinergic function contributes to successful CPFE performance and subsequently to impaired neuronal plasticity that results in *Egr-1* dysregulation will need to be determined in future studies.

The current dissertation proposes a mechanism by which contextual learning may develop and be disrupted following neonatal alcohol exposure. In addition to novel insights into the ontogeny and neurobiological substrates of the contextual learning, this dissertation has provided a foundation upon which future research on FASD can be based in order to advance our understanding of the detrimental effects of neonatal alcohol on developing brain.

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APPENDIX A

CANNULA PLACEMENTS FOR EXPERIMENT 9.2A-C

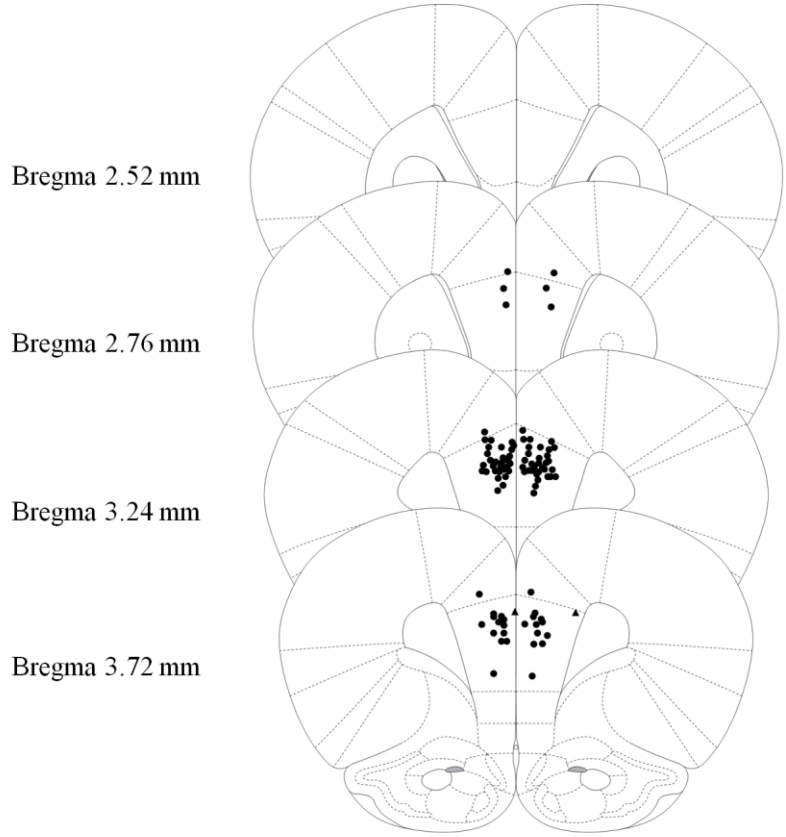

Figure A.1 Schematic representation of injection cannula tip placement in the mPFC for Experiment 3.2a. Animals included in final analyses are represented by filled black circle while animals excluded as mPFC placement misses are filled black triangle. Extremely anterior (Bregma 4.68mm or more) or posterior (Bregma 2.28 or less) were automatically excluded and are not represented in the following figure. Coronal brain images are adapted from the rat brain atlas of Paxinos and Watson Paxinos and Watson (2013).

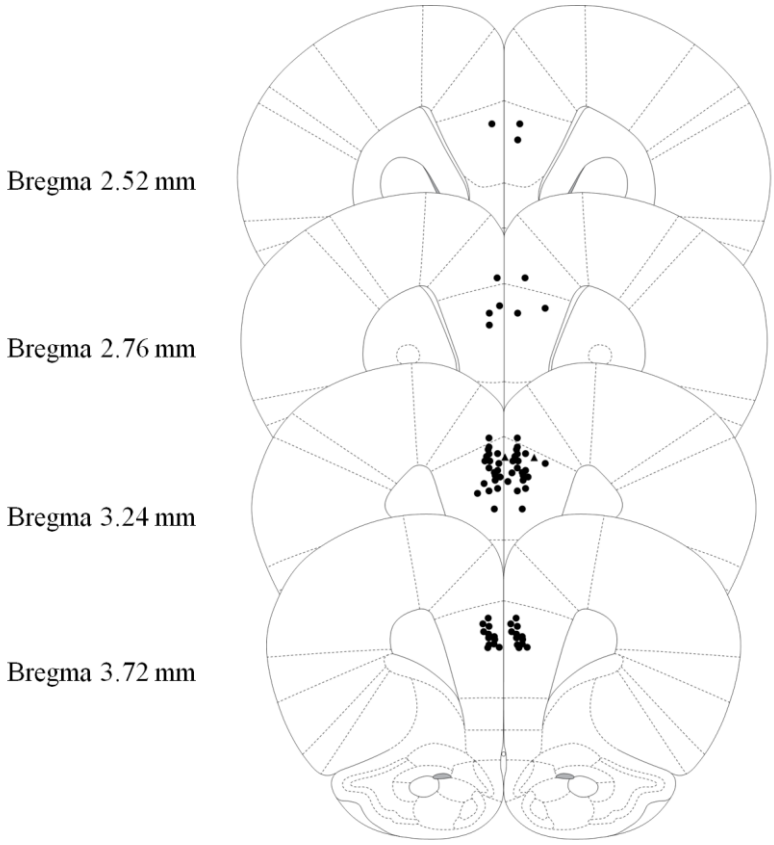

Figure A.2 Schematic representation of injection cannula tip placement in the mPFC for Experiment 3.2b. Animals included in final analyses are represented by filled black circle while animals excluded as mPFC placement misses are filled black triangle. Extremely anterior (Bregma 4.68mm or more) or posterior (Bregma 2.28 or less) were automatically excluded and are not represented in the following figure. Coronal brain images are adapted from the rat brain atlas of Paxinos and Watson (2013)

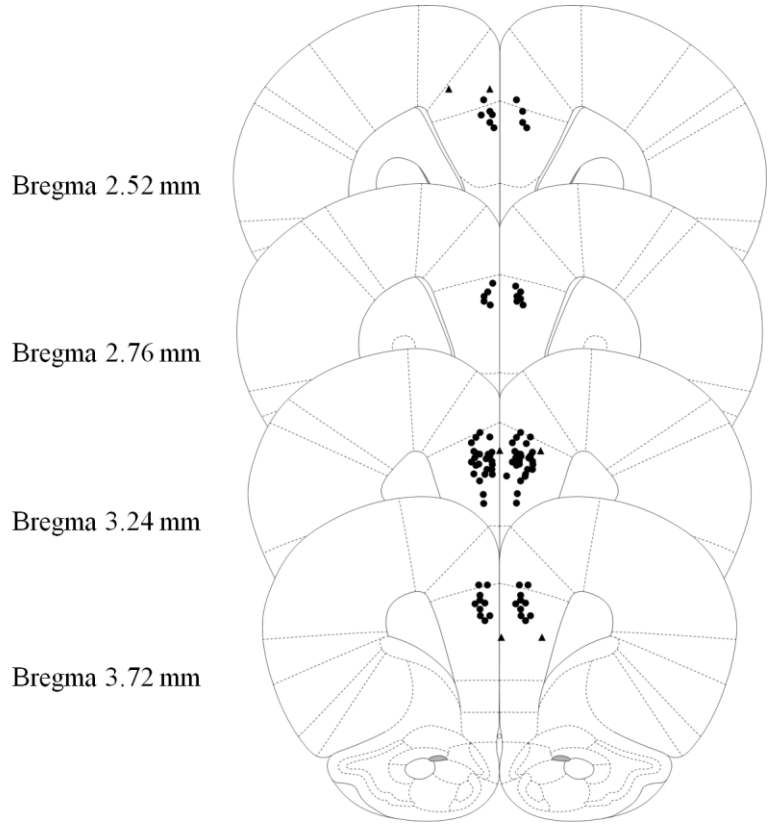

Figure A.3 Schematic representation of injection cannula tip placement in the mPFC for Experiment 3.2c. Animals included in final analyses are represented by filled black circle while animals excluded as mPFC placement misses are filled black triangle. Extremely anterior (Bregma 4.68mm or more) or posterior (Bregma 2.28 or less) were automatically excluded and are not represented in the following figure. Coronal brain images are adapted from the rat brain atlas of Paxinos and Watson (2013).

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

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Application to Use Animals in Research
(New and 3-Yr submission)

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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 **Genus Species:** *Rattus norvegicus*	
Date of Submission: 4/7/2017	

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Date of Approval: 6/1/17

Rev. 03/2016 Page 1 of 49

APPENDIX C

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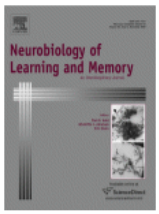

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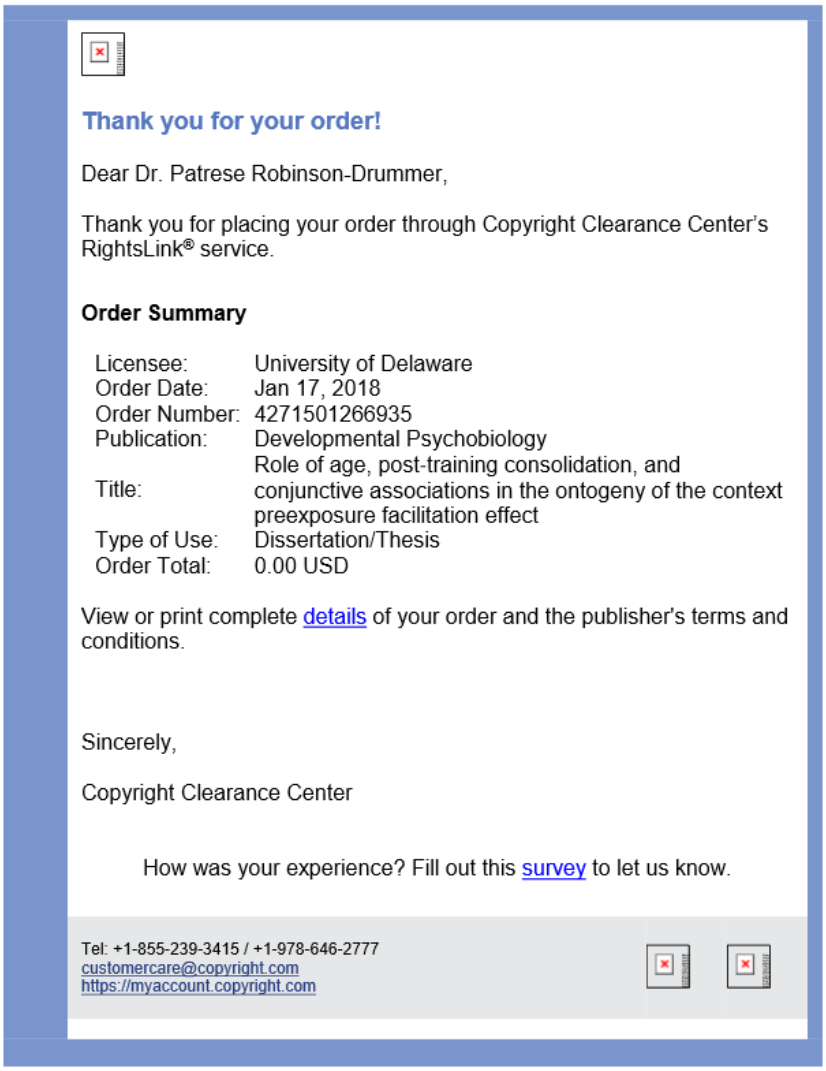

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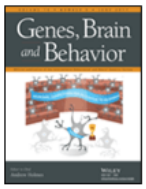

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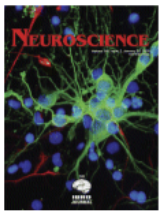

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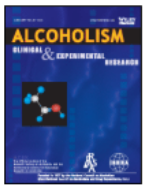

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