ONTOGENY AND NEURAL SUBSTRATES OF THE CONTEXT PREEXPOSURE FACILITATION EFFECT ON CONTEXTUAL FEAR CONDITIONING

by

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ABSTRACT

The essential mnemonic role of the hippocampus in contextual fear conditioning has been reliably demonstrated in the intact adult rat, and is believed to emerge around post-natal day (PND) 23. The mnemonic role mediates the conjugation of the individual feature representations of the context into a unified conjunctive representation, which can then be associated with the reinforcer. However, there is evidence that conditioning at the PND 23-24 may be typically supported by a feature-based simple associative system (SAS) that is hippocampus-independent. To address this issue, a variant of contextual fear conditioning that favors utilization of the hippocampus-dependent configural associative system (CAS) while minimizing contributions from the hippocampus-independent SAS was implemented early in ontogeny. This variant, termed the context-preexposure-facilitation-effect-of-the-immediate-shock-deficit (CPFE-ISD), involves exposure to context and foot shock on successive occasions. After various training parameter manipulations, the ability for the hippocampus-dependent variant seems to emerge rapidly between PND 17 and PND 24, and continues to develop to PND 31. Additional evidence suggests the mnemonic function at PND 24 is mediated by hippocampal long-term potentiation as antagonism of NMDA-type glutamate receptors in the dorsal hippocampus with dizocilpine blocked conditioning in the variant paradigm. This ontogenetic profile of the CPFE-ISD parallels that for conventional context conditioning, suggesting common neural substrates may control conditioning in both paradigms. This is important because the behavioral and neural

mechanisms of conventional context conditioning are more variable and controversial than of the CPFE-ISD. Previously mentioned support for SAS-mediated conditioning at PND 23-24 may have been due to the protracted development of the SAS-inhibitory function of the CAS, relative to the mnemonic function, which clearly emerges by PND 24.

Chapter 1

GENERAL INTRODUCTION

Fear Conditioning

In Pavlovian or classical fear conditioning, an emotionally neutral stimulus, the conditioned stimulus or "CS" (e.g., a light or tone) is presented so that it precedes and overlaps a biologically significant stimulus, the unconditioned stimulus or "US" (e.g. foot-shock). After a relatively low number of pairings, the presentation of the CS alone (without subsequent US) elicits an emotional and behavioral response (the conditioned response or "CR"). For rats, one CR is a species-typical response, termed *freezing*, that is characterized by the cessation of all movement except respiration. The level of fear being expressed corresponds with the amount of freezing behavior observed. Interestingly, this freezing behavior can be elicited merely by reintroducing the rat to the context in which the shock US was presented, so that fear expression does not always require the presentation of any explicit CS (tone or light). In this case, the environment comes to predict the aversive US, and this conditioning may even occur after only a single presentation of the US. This type of conditioning is termed contextual fear conditioning. Many of the underlying neural mechanisms mediating these two types of fear conditioning---discrete-cue versus contextual---are believed to overlap, while others do not.

Neural Substrates of Fear Conditioning

In general, fear conditioning requires the proper functioning of the amygdala. As summarized in Figure 1 (from Maren, 2001), the basolateral complex of the amygdala (comprised of the lateral, basolateral and basomedial nuclei) is of particular importance and has been called the "neuroanatomical hub for learned fear" (Fanselow & Poulos, 2005) and the "locus for the formation and storage of CS-US associations during Pavlovian fear conditioning" (Maren, 2001). Sensory information regarding potential CSs arrives at the lateral nucleus of the amygdala through a variety of pathways, depending upon the complexity the stimulus. The best-characterized CS pathway concerns the relay of a simple auditory or tone CS, which is commonly used in discrete-cue fear conditioning. First, the sound wave is detected and transduced into electrical signals by the auditory sense organs. This nervous auditory information is then transmitted through the auditory system to the medial geniculate nucleus of the thalamus and then relayed directly to the lateral nucleus of the amygdala (Fanselow & Poulos, 2005. Furthermore, the basolateral complex of the amydgala (BLA) receives US information through a variety of routes, including direct projections from posterior thalamus and insular cortex. The convergence of CS and US information at the BLA suggests it is responsible for the formation of the CS-US association. The association is established via the increase in synaptic efficacy of the CS-pathway onto the cells of the BLA commonly activated by the US-pathway. This basic cellular model of Pavlovian conditioning culminates with the CS-pathway being able to activate the BLA in a manner that produces a behavioral response similar to the one resulting from activation by the US-pathway. Arguably, the increased CS-pathway synaptic strength arises primarily by the mechanism of long-term potentiation (LTP). LTP is a neuromolecular process induced by the activation of the N-methyl-D-aspartate

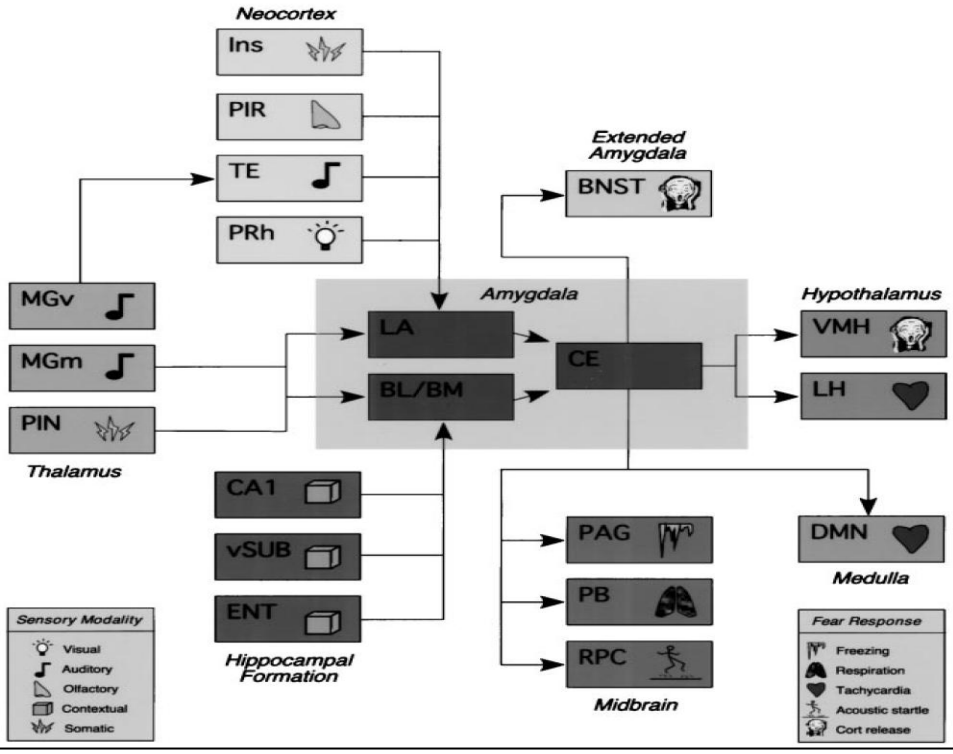

Figure 1. Anatomy of Fear Conditioning Circuits in the Brain.

The amygdaloid nuclei (shown in the center) can be roughly divided into two subsystems. These include the lateral (LA), basolateral (BL), and basomedial (BM) nuclei, which together for the basolateral complex (BLA) and the central nucleus (CE). The BLA reveives and integrates sensory information from a variety of sources. These include the medial and ventral divisions of the thalamic medial geniculate nucleus (MGm and MGy, auditory), the perirhinal cortext (PRh, visual), primary auditory cortex (TE), the insular cortex (INS), gustatory and somatosensory), the thalamic posterior intralaminar nucleus (PIN, somatosensory), the hippocampal formation (spatial and contextual) including area CA1, the ventral subiculum (vSUB), the entorhinal cortex (ENT) and the piriform cortex (PIR, olfactory). Thus, the BLA is a locus of sensory convergence and a plausible site for CS-US association to the CE, where divergent projections to the hypothalamus and brainstem mediate fear responses such as freezing (periaqueductal gray, PAG), potentiated acoustic startle (nucleus reticularis points caudalis, RPC), increased heart rate and blood pressure (lateral hypothalamus, LH; dorsal motor nucleus of the vagus, DMN), increases respiration (parabrachial nucleus, PB), and glucocorticoid release (paraventricular nucleus of the hypothalamus, PVN; bed nucleus of the stria terminalis, BNST). For simplicity, all projections are drawn as unidirectional connections, although in many cases these connections are reciprocal.

Figure and caption taken directly from Maren (2001)

(NMDA)-type glutamate receptors which begin an intracellular cascade of events that eventually increase the presynaptic release and/or postsynaptic responsiveness to glutamate (Fanselow & Poulos, 2005). Postsynaptic response may be enhanced by the addition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, whose activation generates excitatory postsynaptic potentials by increasing the flow of cations across the membrane. Once a strong association has been established, the conditioned freezing response to the CS is generated through the output of the BLA onto the central nucleus of the amygdala, which then projects to the ventral periaqueductal gray, the area responsible for the production of the observable defensive behaviors (Kim et al., 1993; Fanselow & Poulos, 2005). More complex CSs require higher order processing and alternative CS pathways are used. However, these alternate CS pathways still terminate at the BLA; the neural hub for the association of the CS with the US. Successful fear conditioning to the context then, requires the relay of contextual information to the BLA for the context-shock association to be formed. Under normal circumstances, the dorsal hippocampus and adjacent medial temporal lobe structures (entorhinal, perirhinal, postrhinal cortices) seem to be critical sites for the processing of contextual cues, and this information is then relayed to the BLA via the subiculum coursing through the ventral angular bundle (Phillips & LeDoux, 1992; Fanselow & Poulos, 2005).

A variety of lesion studies provided substantial evidence implicating the roles of these specific neural structures in fear conditioning. Bilateral electrolytic lesions performed before training (*anterograde*) on the amygdala and hippocampus afforded initial insight concerning the differential roles the two structures played in discrete-cue (tone) and contextual fear conditioning. Lesions of the amygdala

abolished conditioning for both paradigms, while lesions of the dorsal hippocampus (DH) only produced deficits in contextual fear conditioning. The general role of the amygdala in fear conditioning was consistent with an extensive preexisting literature, while the apparent role of the hippocampus in contextual fear conditioning was a significant discovery (Phillips & LeDoux, 1992). Electrolytic lesions of the DH at various intervals after training (*retrograde*) revealed its time-limited role in the expression of contextual fear. An intact hippocampus was required for fear to be expressed a day after training, but not 28 days later. Furthermore, these retrograde lesions of the hippocampus had no effect on auditory discrete-cue fear conditioning. Therefore, the researchers believed the hippocampus only to be involved in the storage of the context-shock associative memory, and this involvement is transient insomuch that it is assumed by other structures over time (Kim & Fanselow, 1992). Neurotoxic lesions (achieved by infusing sufficient levels of NMDA to result in excitotoxic neuronal death) of the DH further illuminated its role in contextual fear conditioning. Whereas electrolytic lesions damage both neurons and fibers of passage in the area of the lesion, neurotoxic lesions specifically target neuron cell bodies but spare the axonal fibers passing through the particular region. Anterograde neurotoxic lesions of the DH neurons *did not* affect contextual fear conditioning, while retrograde lesions again produced time-limited deficits. As a result, the neurons of the dorsal hippocampus only seem to be *required* for the temporary expression but not acquisition of contextual fear (Maren et al., 1997). In contrast with Phillips & LeDoux (1992), anterograde electrolytic lesions of the DH were also found to reduce conditional freezing following auditory-cue fear conditioning, possibly by interfering with test performance (Maren et al., 1997).

The Two-Process Model of Contextual Fear Conditioning

Extending upon Sutherland and Rudy's configural learning theory, Rudy & O'Reilly (2001) begin to describe, and with further elaboration by Rudy et al. (2004), a *two-process model* in which two distinct and competing associative systems can be used to mediate fear conditioning to the context. A similar model has been detailed by a number of other researchers (Fanselow, 2000) and is depicted in Figure 2 (Maren, 2001). Both systems are capable of representing the context (*dual representations*) in different although sufficient manners for the association with the shock. The first is a simple associative system (SAS) where individual sensory elements or features of the context can be associated with the US (*features view*). The underlying neural mechanisms of this strategy are likely comparable to the aforementioned simple auditory CS pathway and require minimal higher order processing (the exact pathway depends on the modality of the elemental CS). Citing work from Nadel & Willner (1980), neocortical systems seem capable of representing those individual features of the context as the basis for feature-to-feature association (Rudy & O'Reilly, 2001). Hypothetically, if the SAS is utilized for conditioning to a single feature, the presentation of that particular individual cue (e.g. color of the context) alone and apart from the other contextual features (e.g. tactile sensation of metal rods, odor of context, background noise) would invoke a fear response that is identical to the one that would be elicited upon reintroduction to the entire shock context itself. That is, since the animal only associates a particular individual feature of the context with the shock, behavioral fear responses result solely from the perception of that feature; the other additional characteristics of the testing context are irrelevant so long as the particular individual feature is present and perceivable.

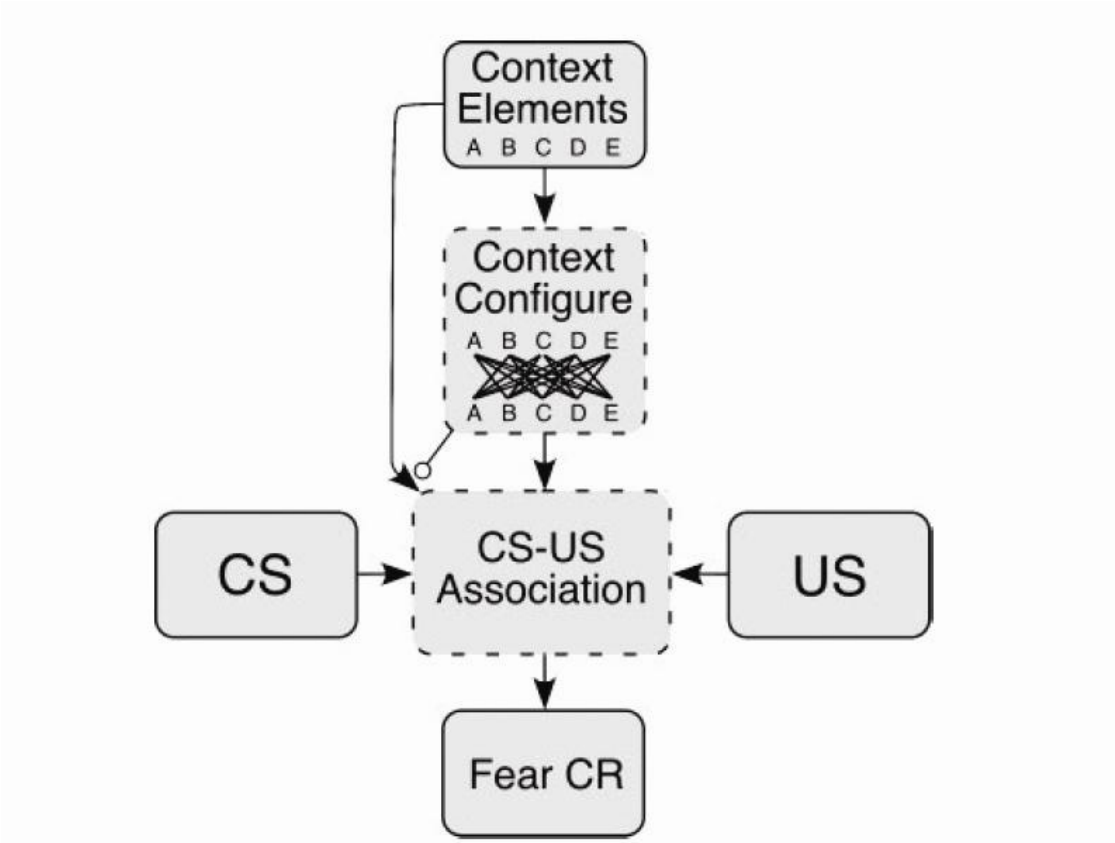

Figure 2. Schematic of the Two-Process Model

Two distinct and competing associative strategies can be used for successful contextual fear conditioning. The individual contextual elements can be associated with the shock US via the simple-associative system (SAS). Alternatively, the contextual elements can be conjugated into a unitary contextual representation by the configural-associative system (CAS), and the context configure may then enter into association with the shock. Both systems can sufficiently support conditioning, but the CAS is believed to competitively inhibit the function of the SAS (open white circle) and dominate conditioning in the intact rat.

Figure and caption taken from Maren (2001) with slight modification

However, contextual fear conditioning supported by the SAS probably results from the simultaneous processing of many of the independent features across all of the sensory modalities. In this view, each feature may be independently

associated with the US and exposing the animal to each feature individually would produce variable levels of freezing across tests, depending upon the strength for each association. More importantly, some measure of fear would be observed for each test. Additionally, if these feature-based fear responses were additive, exposure to all the features simultaneously would elicit an enhanced fear response when compared to the individual feature tests.

The other associative technique is mediated by the higher order configural associative system (CAS). The CAS links together the separate elements of the context and forms a novel representation that codes their conjunction (*conjunctive view*). This idea of a conjunctive representation has also been referred to as a *cognitive map* (O'Keefe & Nadel, 1978), a *unitary representation* (Fanselow, DeCola, & Young, 1993) (cited in Rudy & O'Reilly, 2001), a *unified representation* (Anagnostaras et al., 2001) or an *integrated "Gestalt" representation* (Fanselow, 2000). This particular CAS is believed to support *rapid* conjunctive learning that *automatically* occurs simply as a consequence of the rat's active *exploration* of the environment. Once formed, the unitary conjunctive representation of the context can then be associated with the US via Pavlovian mechanisms. Under normal circumstances, the CAS is also assumed to competitively inhibit the function or utilization of the SAS and dominate during the acquisition of contextual fear. Thus, the CAS has two independent roles: a mnemonic one (formation of conjunctive representation) and an inhibitory one (Fanselow, 2000; Rudy & O'Reilly 2001; Rudy et al., 2004). In contrast to the pattern of observed fear responses after SAS-mediated contextual fear conditioning, utilization of the CAS would result in significant fear responses only after reintroduction to the entire shock context, while low levels of fear expression would

be observed following tests of the individual features (because the CAS inhibits the SAS and these "elemental associations" were never formed).

The Hippocampus and Contextual Conjunctive Representations

The hippocampus has been accepted to be an essential neural structure in certain forms of learning and memory. In humans, it has been argued to mediate the formation of memories encoding everyday facts and events that are available for conscious recall, so-called *declarative memories* (Squire, 1992). The observation of hippocampal "place cells" (pyramidal cells that exhibit location-specific activity) led some researchers to implicate the hippocampus in *spatial memory*, specifically in the formation of *cognitive maps* and their use in navigation through space (O'Keefe & Nadel, 1978, cited in Morris, 2008) Consistent with this notion, the importance of the hippocampus has been demonstrated in a variety of spatial cognitive tasks, such as the radial maze task (Olton and Samuelson, 1976), the Morris hidden water-maze task (Morris, 1981), and spatial delayed alternation (Green & Stanton, 1989; Watson et al., 2009). To construct these spatial maps, the variety of perceived elemental sensorial features must be conjugated or interconnected into a unitary, integrated, configural, and/or relational representation of the entire environmental context (O'Keefe & Nadel, 1978; Rudy & O'Reilly, 2001; Fanselow, 2000).

With respect to contextual fear conditioning, a number of researchers have argued that the hippocampus mediates the formation of the conjunctive representation (Fanselow, 2000; Maren et al. 1997; Rudy & O'Reilly, 2001) with the preponderance of lesion data being presented as evidence for this mnemonic function. The findings that damage to the hippocampus impairs contextual fear conditioning without affecting auditory-cue fear conditioning demonstrate the existence of both hippocampal-

dependent and hippocampal-independent memories within the same preparations. As contextual fear conditioning is believed to require the rat to associate the shock with a representation of the context, the hippocampus seems to be involved at some level with the contextual representation. The demonstrations of a temporally graded retrograde amnesia for contextual fear following both electrolytic (Kim & Fanselow, 1992) and neurotoxic (Maren et al., 1997) lesions indicate the hippocampus is required for the formation, transient storage and/or consolidation of the contextual memory, but not the retrieval of old context memories that are stored in other neural areas (Fanselow, 2000). Additionally, the apparent discrepancies following the different anterograde lesion techniques offer further clues about the neural basis of contextual conditioning. Since neurotoxic lesions spare the fibers of passage, while electrolytic lesions do not, those fibers must be important at some point during the acquisition or expression of contextual fear. Specifically, the destruction of the projections from the ventral subiculum to the nucleus accumbens is thought to cause the observed deficits in contextual fear conditioning following the anterograde electrolytic lesions. Lesions of this subiculo-accumbens pathway prolong exploratory behavior and/or cause locomotor hyperactivity; consequences that may be interfering with the mnemonic benefits of exploration (i.e., the formation of the contextual representation) and test performance (freezing behavior), respectively (Fanselow, 2000; Maren et al. 1997). Meanwhile, neurotoxic lesions of the dorsal hippocampus do not cause an increase/prolonging of exploratory behavior when compared to shams, so interference with freezing performance cannot explain the *retrograde* amnesia observed (Fanselow, 2000). To explain the lack of *anterograde* amnesia following neurotoxic lesions (where lesions before training did not impair contextual fear conditioning), one must

realize that rats have both hippocampal-dependent and independent fear associative mechanisms. Generally successful auditory-cue fear conditioning following both anterograde and retrograde lesions (Phillips & LeDoux, 1992; Kim & Fanselow, 1992) demonstrates the rats' ability to acquire fear memories independent of the hippocampal memory system (Fanselow, 2000). Most importantly, successful contextual fear conditioning following anterograde neurotoxic lesions suggests a similar hippocampal-independent system may be utilized to acquire contextual fear, but only when the cells of the dorsal hippocampus are lesioned prior to conditioning (evidenced by the observed retrograde amnesia). As demonstrated in Figure 3, the previously described *two-process model* parsimoniously explains such patterns of behavioral data if the CAS is believed to be hippocampal-dependent while the SAS is hippocampal-independent. In this view, lesions of the hippocampal cells before conditioning free the SAS from the CAS's competitive inhibition, allowing the SAS to mediate the acquisition of contextual fear and anterograde amnesia is not observed. However, if the hippocampus is intact during conditioning, both the mnemonic and SAS-inhibitory functions of the CAS are employed. Since the CAS was utilized during conditioning, short-interval retrograde lesions of the hippocampus impair the retrieval of the contextual representation required to activate the context-shock associative memory stored in the amygdala, and since the SAS was also inhibited during conditioning, retrograde amnesia is observed (Fanselow, 2000; Maren et al. 1997, Rudy & O'Reilly, 2001).

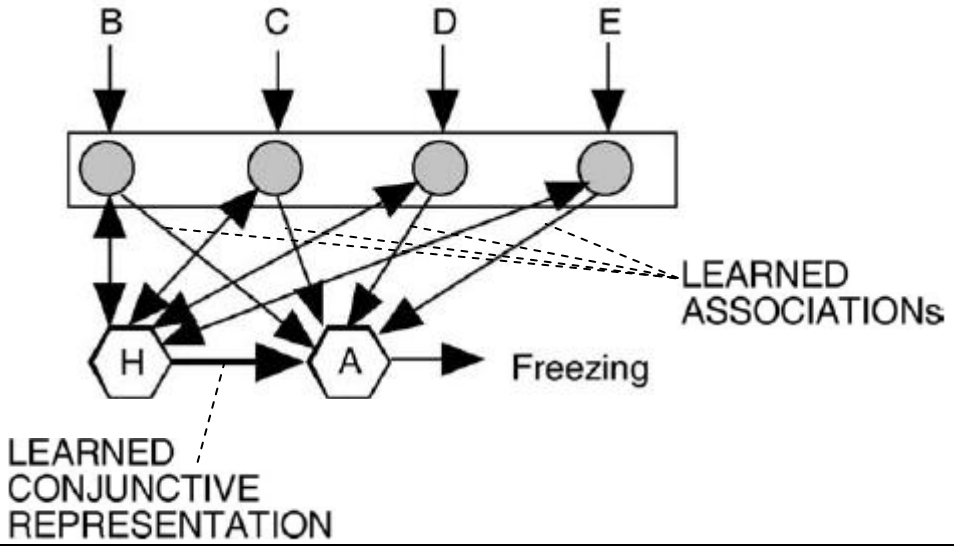

Figure 3. Neural Substrates of the Two-Process Model

The individual features (B, C, D, and E) are perceived and represented in the cortex (gray circles). Reciprocal cortico-hippocampal connections allow the hippocampus (H) to (1) conjugate the feature representations into the conjunctive contextual representation and (2) inhibit individual features associations. The amygdala (A) is the neural site that associates either the conjunctive representation or the individual features with the shock. Once an association is established, activation of that particular contextual representation (conjunctive or features) consequently activates the shock memory and the amygdala generates the emotional fear state, including the observed freezing behavior.

Figure and caption adapted from Rudy et al. (2004)

The Context Preexposure Facilitation Effect

Low levels of contextual fear conditioning have been observed after a variety of experimental manipulations. For example, low levels of fear conditioning occurs if rats are isolated immediately after conditioning (Pugh et al., 1999; Rudy, 1996), if they are conditioned at noon as opposed to the early morning or late afternoon (Rudy & Pugh, 1998), if post-training injections of morphine are administered (Rudy et al., 1999), if they are adrenalectomized prior to training (Pugh

et al., 1997), or if juvenile rats are tested ten minutes after conditioning (Rudy & Morledge, 1994; cited in Rudy & O'Reilly, 2001). In each of the aforementioned cases, exposing the rat to the conditioning chamber so that it can freely explore the training context before and on a separate occasion from training (termed *preexposure*) restored conditioning to control levels (Rudy & O'Reilly, 2001). This enhancement of previously low levels of conditioning following preexposure (typically a day before conditioning) has been termed the *context preexposure facilitation effect* (CPFE) (Rudy et al., 2004). Experimentally, the *general* CPFE is observed by reporting higher levels of conditioning for the group preexposed to the training context (*preexposed-same*) relative to the control group, which usually is preexposed to another distinctly different context (*preexposed-other*) to control for handling effects. In general, the facilitating effect of context preexposure is believed to depend upon the formation of the conjunctive contextual representation during that preexposure period (Fanselow, 2000; Rudy et al. 2004). Successful formation of the conjunctive representation can only occur if the entire set of contextual features is experienced simultaneously (*conjunctive view*). If this is so, then separate preexposures to the individual features that make up the context should not facilitate conditioning. However, if the CPFE results from preexposure strengthening the individual feature representations that are then associated during training (*enhanced saliency view*), then the features do not have to be sampled simultaneously, they just all need to be experienced (Rudy & O'Reilly, 2001). Using manipulations of preexposure condition such as preexposing rats to the entire training context vs. the separable features and/or combinations of the features, Rudy & O'Reilly (1999) provide substantial support for the *conjunctive view* of the CPFE . The methodology and results for one variation of the experiment are depicted

in Figure 4. Animals preexposed to the entire, unified context demonstrated significant levels of contextual fear conditioning, while feature preexposures resulted in comparatively lower levels of fear expression. The feature group demonstrated conditioning levels that did not differ significantly from the control group that was preexposed to a context not sharing any features with the training context (Rudy & O'Reilly, 1999).

Although the results of Rudy & O'Reilly (1999) were consistent with the *conjunctive view* of the CPFE, the feature preexposures did result in *some* level (albeit significantly lower levels) of contextual fear conditioning. Furthermore, even though the features group did not differ from the control group (effectively challenging the *enhanced saliency view* of the CPFE), the minor levels of conditioning generated for both groups may still have been mediated by the hippocampus-independent SAS. Once more, the hippocampus-dependent CAS is believed to be involved in conditioning to the conjunctive representation, while the hippocampus-independent SAS mediates feature-based associative learning. As some measure of fear was observed following feature preexposures (and in the control group), any contributing involvement of the SAS in the observed high levels of conditioning for the group preexposed to the entire context cannot be confidently ruled out. However, Rudy & O'Reilly (2001) describe a contextual fear conditioning protocol (the CPFE-ISD paradigm) that is believed to selectively favor the utilization of the hippocampus-dependent CAS while minimizing the potential contribution from the hippocampus-independent SAS. The key component to the paradigm is the observation of the *immediate-shock deficit* (ISD) that was first described by Fanselow (1986).

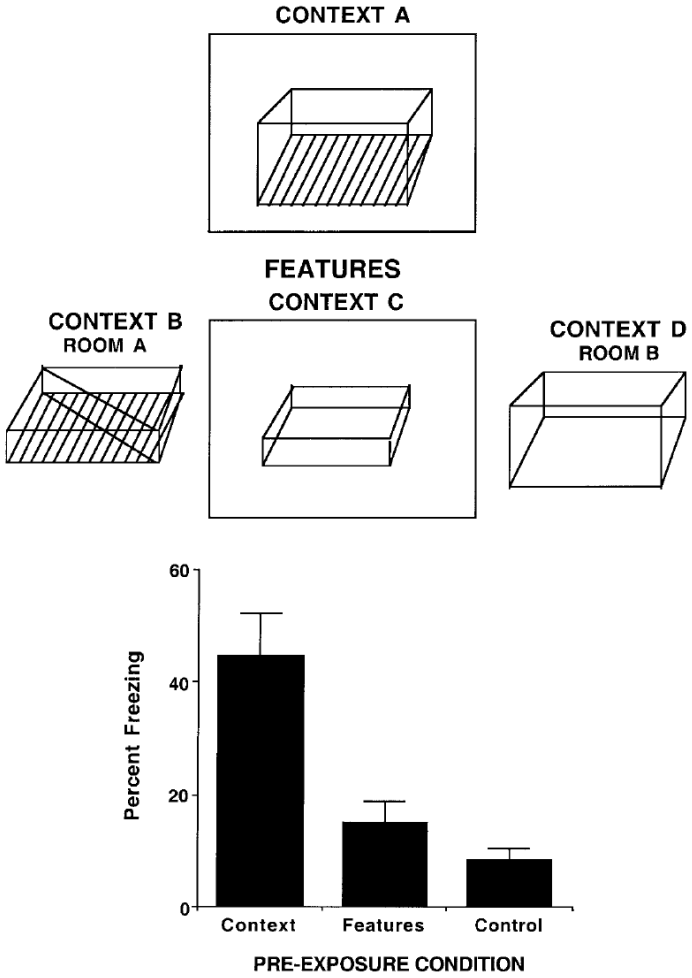

Figure 4. Evaluation of the Conjunctive View vs. the Enhanced Saliency View of the Context Preexposure Facilitation Effect

The top of the figure illustrates the methodology used to expose rats to the entire training context (Context A) or to the un-conjoined features that made up Context A (Contexts B, C and D). The rats in the context preexposure condition were preexposed to Context A, whereas the rats in the features preexposure conditioning were exposed to Contexts B, C, and D. The rats in the control condition were exposed to a different context (a mouse cage) not represented in this figure. The bottom of this figure presents the mean percentage of freezing as a function of preexposure conditions just described. Bars represent standard errors of the mean. Conjunctive view of the CPFE supported as context preexposure resulted in significantly higher levels of conditioning than preexposure to the dissociated features, which did not enhance conditioning relative to the control

Figure taken directly and caption adapted from Rudy & O'Reilly (2001)

Context Preexposure Facilitation Effect Eliminating the Immediate Shock Deficit

Fanselow (1986) found that if a rat is shocked immediately upon its first placement into the chamber, it will exhibit markedly low levels of conditional freezing when tested later (the ISD). The strikingly low levels of conditioning following an immediate shock suggest that both the SAS and CAS could not function under those training conditions because of insufficient processing time. In a study administering shocks after various *placement-to-shock intervals* (PSI: the time separating insertion into the conditioning chamber and delivery of the shock), Fanselow (1986) demonstrated the first ever reported CPFE. However, in this original demonstration, a significant CPFE was only observed when the PSI during training was *at least* 8 seconds, so that the ISD (< 8 sec) *was not* alleviated by context preexposure (Fanselow, 1986). Fanselow (1990) believed that during training, the preexposed animals needed a brief period of re-exposure to the context (lasting a minimum of approximately 8 seconds) prior to shock delivery so that the conjunctive representation (formed during preexposure) could be successfully retrieved and readily available for association with the shock. Therefore, the lack of a CPFE for the immediately shocked rats was due to the failed retrieval of the context memory before the shock was experienced so that the context-shock association could not be formed, resulting in low levels of fear expression. However, the timely retrieval of the conjunctive memory before training can be facilitated with retrieval cues.

Rudy & O'Reilly (2001) detail a methodology to establish the characteristics of the transport container as retrieval cues for the conjunctive representation, so that the ISD can be ameliorated via the CPFE. Transporting the rat multiple times to the preexposed context within the same transport container (they used a black ice bucket) creates an association between the features of the transport

container and the conjunctive memory of the preexposed context. Once the transport features-context memory association is established, exposure to the transport features cues the retrieval and subsequent activation of the associated conjunctive representation through the process of *pattern completion* (Rudy& O'Reilly, 2001; Rudy et al., 2004). As the contextual memory is activated during transport, it is readily accessible for the BLA to form the association between it and the immediately delivered aversive shock stimulus. Consistent with this theoretical framework, multiple preexposures resulted in a significant CPFE even when trained with an immediately delivered shock (Rudy & O'Reilly, 2001). The specific context preexposure facilitation effect that abolishes the *immediate shock deficit* shall be termed the CPFE-ISD throughout this thesis.

Rudy & O'Reilly (2001) also provide evidence to support the *conjunctive view* of the CPFE-ISD. In a within subjects design, all of the rats were preexposed to the conditioning chamber via transport in the black ice bucket to establish a specific transport-preexposed context association. The same rats were also transported and preexposed to another context via a different transport container (mouse cage). The within subjects design ensured every rat had the opportunity to acquire the conjunctive representation of the conditioning chamber, but uniquely established the black ice bucket as the retrieval cue for that preexposed context memory. On the training day, rats were transported in either the black ice bucket or the mouse cage to the conditioning chamber and given an immediate shock. If preexposure merely strengthened the individual feature representations to subsequently facilitate SAS-mediated conditioning to those features (consistent with the *enhanced saliency view* of the CPFE), then both groups would demonstrate some level of fear expression during

the test session as every rat was preexposed to the training context. However, only the rats transported in the black ice bucket exhibited high levels of freezing, while the control group displayed *very little* freezing, i.e., the ISD (Rudy & O'Reilly, 2001). These results suggest that for a successful CPFE-ISD to observed, the conjunctive contextual representation must be constructed by the CAS during preexposure, and this conjunctive representation must be successfully retrieved via transport cue facilitation prior to the delivery of the immediate shock.

The Hippocampus and the Context Preexposure Facilitation Effect

The numerous lesion studies previously described (Phillips & LeDoux, 1992; Kim & Fanselow, 1992; Maren et al., 1997) began unveiling the role of the hippocampus in standard contextual fear conditioning, where PSIs are longer and no preexposure was given. However, since extrahippocampal regions can support feature-based contextual fear conditioning, proper investigations into the role of the hippocampus require a methodology that depends upon the formation of the conjunctive representation for successful contextual fear conditioning to occur. This can be achieved by forcing the rat to learn about the context on a separate occasion from when associations are formed with the shock, as in the CPFE-ISD paradigm. Using the CPFE-ISD paradigm, Rudy, Barrientos, & O'Reilly (2002) provided strong support for the mnemonic role of the hippocampus in the formation of the conjunctive contextual representation. Neurotoxic lesions of the dorsal hippocampus (DH) before the multiple preexposures (anterograde) eliminated the CPFE. Lesions cannot be used to examine the contributions of the hippocampus across the three phases of the CPFE-ISD paradigm, but pharmacological manipulations that temporarily inactivate the structure can. Hence, Matus-Amat et al. (2004) infused 5-aminomethyl-3-

hydroxysoxazole (muscimol), a $GABA_A$ agonist that potentiates inhibitory synaptic transmission, to inactivate the DH across the various stages of the CPFE-ISD paradigm. Inactivating the DH before each individual phase (preexposure, immediate shock training, or testing) or across all three phases (to address state-dependent learning hypothesis) significantly reduced freezing levels when compared to vehicle controls. Meanwhile, infusions of muscimol immediately after context preexposure (allowing the formation of the conjunctive representation during preexposure) and before the initiation of a standard conditioning protocol where the shock was delivered after a long PSI (120s in this case) upon the first introduction to the conditioning chamber (i.e., no preexposure phase) had no effect on conditioning levels. The latter two manipulations verify the reported impairments by muscimol cannot be attributed to a performance deficit, as freezing behavior could still be observed following infusion of the drug. Therefore, the hippocampus seems to be required for (1) the formation of the conjunctive contextual representation during the preexposure phase, (2) the retrieval of the context memory so that it may be associated with the shock during the training phase, and (3) the retrieval of the context memory for the activation of the context-shock association that produces the fear behavior observed during the testing phase (Matus-Amat et al., 2004).

Disruption of overall activity, by muscimol inactivation or neurotoxic lesion, blocks both the mnemonic and SAS-inhibitory functions of the hippocampus. Since SAS-inhibition is reduced, the SAS can form feature-based associations in the standard (or conventional) conditioning protocol to compensate and subsequently mediate contextual fear conditioning (Maren et al., 1997; Matus-Amat, 2004). However, low levels of expressed fear following anterograde neurotoxic DH lesions

(Rudy, Barrientos, O'Reilly, 2002) and muscimol infusion (Matus-Amat, 2004) indicate the hippocampus-independent SAS alone cannot support contextual fear conditioning in the CPFE-ISD paradigm; a *very* important observation. SAS-mediated conditioning requires a fair amount of context exposure that *directly* precedes the shock (i.e., on the same occasion); exposure that an immediate shock does not provide. Thus, delivery of an immediate shock during training, as in the CPFE-ISD paradigm, selectively favors CAS-mediated conditioning while minimizing potential SAS contributions.

To investigate the neuromolecular determinants of the general CPFE, Stote & Fanselow (2004) infused the NMDA-receptor antagonist D,L-2-amino-5-phosphovalerate (APV) into the right lateral ventricle prior to the preexposure phase. Infusions of APV effectively eliminated the CPFE when an intermediate PSI (35s) was used, implicating NMDA receptors in the formation of the conjunctive representation. Citing work from Young, Bohenek, & Fanselow (1994) that demonstrated deficits in standard (long PSI, no preexposure phase) contextual fear conditioning following bilateral infusions of APV directly into the DH, Stote & Fanselow (2004) argue the effect of APV administration during preexposure was primarily due to NMDA-antagonism in the hippocampus. As a result, the formation of the conjunctive representation during preexposure is believed to depend upon the function of NMDA-receptors in the hippocampus (Stote & Fanselow, 2004), probably through the mechanism of LTP (Young, Bohenek, & Fanselow, 1994).

Ontogeny of Contextual Fear Conditioning and the Context Preexposure Facilitation Effect

Auditory-cue and standard contextual fear conditioning dissociate during development, where auditory-cue fear conditioning may be observed by post-natal day (PND) 18, contextual fear conditioning begins to emerge around PND 23 (Rudy, 1993). As the hippocampus is believed to play an integral role in contextual but not auditory-cued fear conditioning (Phillips & LeDoux, 1992; Kim & Fanselow, 1992), the ontogenetic dissociation is often attributed to the ongoing maturation of the hippocampus or the protracted emergence of a functional hippocampus-dependent CAS that interacts with the already established amygdala-dependent SAS (Rudy, 1993; Stanton, 2000).

Rudy & Morledge (1994) established an ontogenetic profile of contextual fear conditioning by manipulating the retention interval (the time between the conditioning session and the testing session). Rats conditioned on PND 23 and PND 32 demonstrated increasing levels of contextual fear conditioning as the retention interval increased from 10 min to 24 hours, while PND 18 conditioned rats failed to exhibit fear conditioning across those same retention intervals. Remarkably low levels of freezing were observed for both the PND 23 and PND 32 conditioned rats after a 10 min retention interval, while high levels of fear were expressed when testing occurred after a 24 hour retention interval. Notably, preexposure to the training context 24 hours before conditioning significantly enhanced freezing levels for rats conditioned on PND 24 when tested after that 10 min retention interval (Rudy & Morledge, 1994). A similar CPFE when testing after a 10 min retention interval was also observed for rats conditioned on PND 31, and support for the *conjunctive view* of the CPFE was also reported (see Figure 4 taken from Rudy & O'Reilly, 1999). As preexposure 10 minutes

before training did not facilitate conditioning for the 10 min retention interval, the benefit of preexposure (i.e., the formation of conjunctive representation) is only realized if the contextual memory can be consolidated into a usable form, and this consolidation period is between 10 minutes and 24 hours (Rudy & Morledge, 1994).

Assuming the contextual fear conditioning ability and related CPFE are mediated by the same neural associative mechanisms throughout the rat's life may be problematic. Unlike previous results, Pugh & Rudy (1996) demonstrated a CPFE could be observed for PND 18 trained rats in a quasi-replication of Rudy & Morledge (1994), where rats were trained in a "salient" black context instead of a clear one. However, a subsequent features vs. context preexposure manipulation suggested the CPFE for PND 18 trained animals seemed to be accounted for by the *enhanced saliency view* of preexposure facilitating SAS-mediated feature-based associations, as no significant difference was reported between the conditioning levels for rats preexposed to the individual features versus the entire unified training context. As illustrated in Figure 5, the *enhanced saliency view* was also supported for preexposed animals trained on PND 23, where both the features and the context preexposures resulted in similar levels of fear expression, regardless of the training context color. Based on their results, Pugh & Rudy (1996) argued PND 18 animals have access to the underlying neural substrates required for long-term contextual fear conditioning. However, the hippocampus and related CAS structures are probably not included in those neural substrates as the observed conditioning seemed to be feature-based. Support for the *enhanced saliency view* for PND 23 trained animals suggests that either the CPFE for these animals results from the enhanced functioning of the SAS, or that the neural substrates mediating the SAS-inhibitory functions of the CAS may not

be sufficiently developed. Although support for the *conjunctive view* of the CPFE has been shown in older animals (Rudy & O'Reilly, 1999), the supposed CAS-mediated CPFE on contextual fear conditioning around PND 24 was challenged by Pugh & Rudy (1996). The results of Pugh & Rudy (1996) suggest that either contextual fear conditioning is supported by the SAS as the ability first emerges, or that the SAS-inhibitory function of the CAS has not sufficiently developed by that age, so that both associative strategies may be simultaneously used and conditioning may result from their concurrent operation.

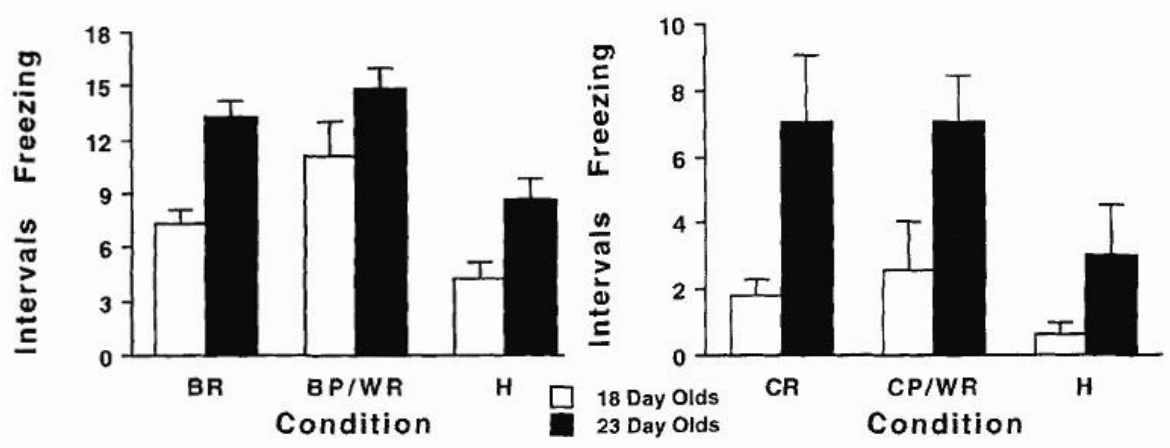

Figure 5. General Support for Enhanced Saliency View of the Context Preexposure Facilitation Effect for PND 23 Trained Animals

Mean number of intervals the freezing response was observed in two contexts as a function of simultaneous or separate preexposure to the elements of the training context. Bars represent standard errors of the mean. BR = black sides and rod floor (training context); BP = black sides and Plexiglas floor; WR = white sides and rod floor; H = handled; CR = clear sides and rod floor (training context); CP = clear sides and Plexiglas floor. Enhanced saliency view of CPFE generally supported for PND 23 trained animals as both preexposure conditions resulted in similarly high levels of conditioning.

Figure and caption adapted from Pugh & Rudy (1996)

It must be noted that all of the aforementioned ontogenetic studies reporting a CPFE used a placement-to-shock interval (PSI) of 120s. As demonstrated in Pugh & Rudy (1996), the potential contribution of the SAS following a long PSI undercuts the assumption that contextual fear conditioning in younger rats depends upon the hippocampus-dependent CAS-mediated construction of the conjunctive contextual representation, as it has rather reliably been shown in adults (Rudy & O'Reilly, 2001; Rudy, Barrientos, & O'Reilly, 2002; Matus-Amat et al., 2004; Stote & Fanselow, 2004). To address this issue, the current thesis used the CPFE-ISD paradigm, which favors utilization of the CAS and minimizes usage of the SAS. Using the CPFE-ISD paradigm, Burman et al. (*under revision*) demonstrated PND 23 preexposed-same animals could successfully condition to fear the context whereas preexposed-other animals could not. This thesis sought to elaborate on the ontogenetic emergence of the specifically *hippocampus-dependent CAS-mediated* variant of contextual fear conditioning by implementing the CPFE-ISD paradigm at various points in ontogeny. Consistent with the general CPFE paradigm, each experiment consisted of consecutive phases: the preexposure phase, the training phase, and the testing phase that were all separated by approximately 24 hours to allow for memory consolidation between phases. Animals were preexposed on either PND 17, 24, or 31 throughout Experiments 1-3. Experiments 1 and 3 trained animals with immediate shock(s) using the CPFE-ISD paradigm, while Experiment 2 varied the PSI to examine the emergence of the general CPFE across ontogeny relative to the standard (longer PSI, no preexposure) form of contextual fear conditioning.

Furthermore, this thesis also hoped to continue the neuromolecular investigations initiated by Burman et al. (*under revision*) of the CPFE-ISD at a point early in ontogeny. Burman et al. (*under revision*) reported systemic dizocilpine (MK-801) administration during the PND 23 preexposure phase impaired the CPFE-ISD relative to saline controls. MK-801 is a potent NMDA-type glutamate receptor antagonist, and these results supported the general role of NMDA-receptors during the incidental formation of the conjunctive representation that occurs while the weanling rat freely explores the context during the preexposure phase. However, Burman et al. (*under revision*) did not examine the contribution of specific neural structures, so the potential difference between weanling and adult neural mechanisms underlying contextual fear conditioning could not be dismissed. Localized MK-801 mediated NMDA-receptor antagonism in the dorsal hippocampus early in ontogeny has impaired performance in other spatial memory tasks that are believed to be hippocampus-dependent (Watson et al., 2009). So, Experiment 4 bilaterally infused MK-801 into the dorsal hippocampus (using the methodology of Watson et al. (2009)) before preexposure on PND 24 to ascertain whether successful conditioning for these younger animals in the CPFE-ISD paradigm was mediated by hippocampal LTP that occurred during preexposure, as it has been demonstrated for adults when using a more general CPFE paradigm (Young et al., 1994; Stote & Fanselow, 2004).

Chapter 2

EXPERIMENT 1

Conventional contextual fear conditioning emerges between PND 17 and PND 23 (Rudy, 1993; Rudy & Morledge, 1994; Stanton, 2000). This experiment hoped to establish a preliminary ontogenetic profile of the CPFE-ISD across this same developmental window. The design was a 2 (preexposure condition: preexposed-same vs. preexposed-other) x 3 (age of preexposure: PND 17, 24, 31) factorial. At each age, rats were exposed to either the training context (preexposed-same) or an alternate context (preexposed-other) for 5 minutes. The following day, to minimize the potential contribution of the SAS, training utilized the delivery of a single shock immediately upon placement into the conditioning chamber (training context) in a manner consistent with the CPFE-ISD paradigm. It is believed that for robust conditioning to be observed, the CAS must create a conjunctive context memory during the preexposure phase that can be retrieved and associated with the immediate shock. Thus, the preexposed-same group should freeze significantly more than the preexposed-other group. This was tested 24 hr later when all groups were exposed for 5 min to the training context. The primary question of interest was whether the CPFE-ISD would emerge between PND 17 and PND 24, as conventional contextual fear conditioning does, or whether its emergence would be delayed, specifically between PND 24 and PND 31.

Methods

The methods for Experiment 1 were as described in my previous research (Burman, Schiffino, Murawski, Rosen & Stanton, *under revision*).

Subjects

Sixty-six Long Evans weanling rats (33 male, 33 female) that were the offspring of 12 different mothers were used in this study. Rats were bred in the University of Delaware Animal Facility from breeders derived from Harlan Long Evans stock. Litters were weighed and culled to eight pups (usually 4 male, 4 female) on post-natal day (PND) 3, and were weaned on PND 21. Dams were housed with their litters in clear Polypropylene containers measuring 8" high x 18" long x 9" wide in a USDA-approved animal facility that was operated according to NIH guidelines. The housing facility was maintained on a 12:12 hour light/dark cycle with lights on at 7 a.m. The age of litters was determined by daily checks during the light part of the cycle with gestational day 22 defined as the day of birth. After weaning from their mothers on PND 21, pups were housed with same sex littermates and continuously supplied with food and water except during experiments. No more than a single same-sex littermate was assigned to a given experimental group, except a single case in which the behavioral data were averaged and included in the analysis as a single observation.

Apparatus and Stimuli

Fear conditioning occurred in 4 medium-sized conditioning chambers (16.5 x 12.1 x 21.6 cm) placed within a fume hood (Rosen et al., 2008).The chambers were made of clear Plexiglas, except for sides facing another conditioning chamber, which were opaque. The bottom of each cage was a grid floor (11.5 cm from top of

chamber) through which foot-shocks were delivered. The grid bars were 0.5 cm in diameter and placed 1.25 cm apart. The US was a 1.5-mA, 2-s, foot-shock delivered by a shock scrambler (Med Associates, Georgia, VT ENV-414S). Movement was recorded and immobility was determined using FreezeFrame software (Actimetrics, Wilmette IL), which measures pixilation changes to assess freezing behavior (Burman et al., *under revision*). The program was set to the 4-chamber/1-camera mode. Preexposure occurred either in these chambers or alternate chambers (see next section). The alternate chambers were 22 x 22 x 26 cm wire mesh cages enclosed in larger sound-attenuated chambers (BRS/LVE, Laurel, MD) lined with sound-absorbing foam (see Brown & Stanton, 2008).

Behavioral Procedure

Consistent with the CPFE-ISD paradigm, the behavioral procedure took place in three phases: preexposure, training, and testing. Rats were preexposed either on PND 17, 24, or 31. Animals were trained the next day (PND 18, 25, or 32) and testing occurred on the day following training (PND 19, 26, or 33). PND24 and 31 rats were weighed and transferred to individual white plastic cages (24 x 18 x 13 cm) two days before the preexposure phase. PND 17 rats remained in their home litter cages. All sessions occurred in the afternoon, beginning between 3:00 and 5:00 pm. Prior to each session, rats were weighed and placed into individual transport containers. Pre-weaned rats were removed from their home litter cages with minimal disturbances. The individual transport containers were 11 x 11 x 18 cm and made of clear lexan. They were surrounded on the outside walls with orange construction paper to make them distinctive and to render all sides opaque. Rats were kept in the transport container while the conditioning chambers were cleaned with 5% ammonium

hydroxide solution before the initiation of each phase. As a result, the rats spent between 2-4 minutes in the transport container before each phase (1 minute during transport to destination, and the remaining time elapsed while chambers were being cleaned). Experimental cohorts were no larger than 24 animals.

For preexposure, rats were run in groups of 2-4 and placed into either the foot-shock chamber where they were to be conditioned the next day (preexposed-same) or a completely separate context (preexposed-other) to control for handling and exposure to novelty. All rats were preexposed to one of the chambers for 5 minutes. The preexposed-other context consisted of a completely different set of chambers in a different room of the building, as described above.

For training, rats were run in pairs matched for age while differing in preexposure condition. Preexposed-same rats were trained in the same chambers they were preexposed to the previous day. A single foot-shock was administered immediately after placement (approx 3-5 s delay). As rats were loaded into the chambers 2 at a time, the first rat loaded received slightly more exposure (2 s). Load order was counterbalanced across preexposure condition and gender. Rats were removed from the chambers as quickly as possible following the foot-shock, returned to their home cages and left for approximately 24 hours until testing.

For testing, rats were run in identical circumstances as for training except that the testing session lasted for 300 s for all animals and no shock was delivered. All rats were tested in the same chamber they were trained in.

Data Analysis and Statistics

The data were initially analyzed using FreezeFrame software (Actimetrics, Wilmette IL) (Burman et al., *under revision*). The bout length was set at 0.75 s and the

freezing threshold was initially set as described in the instructions. A human observer verified the setting by watching the session and adjusting the threshold if necessary to ensure that small movements were not recorded as freezing.

Once percent freezing was determined, the data were imported into Statistica 7 data analysis software for further analysis. A 2 x 2 x 3 factorial ANOVA was run (with sex, preexposure condition, and age of preexposure as independent factors). Eight animals were excluded from the experiment, 1 for procedural error and another 7 were excluded as statistical outliers. A rat was considered an outlier if the absolute value of its Z-score was greater than 1.96. The Z-score was calculated by dividing the deviation of an individual animal's score from the mean of the remaining animals in its group by the standard deviation of those remaining animals. Rats included into the final analyses were distributed across groups as follows: 5 males and 4 females in the PND 17 preexposed-same group, 3 males and 5 females in the PND 17 preexposed-other group, 5 males and 6 females in the PND 24 preexposed-same group, 7 males and 5 females in the PND 24 preexposed-other group, 5 males and 4 females in the PND 31 preexposed-same group, and 5 males and 4 females in the PND 31 preexposed-other group. The number of rats excluded as outliers from these 6 groups, respectively, were: 1, 1, 2, 1, 1, 1. Newman-Keuls post-hoc tests were used to further examine significant interaction trends found in the ANOVA.

Results & Discussion

The results are depicted in Figure 6. There was a trend towards a significant main effect of gender (F (1,46) = 2.96, p<.10), representing slightly greater levels of freezing in males. However, interactions between gender and the other two variables of preexposure age or preexposure condition were not significant (ps>.34).

Significant main effects of preexposure age (F (2,46) = 4.20, p<.05) and preexposure condition (F (1,46) = 13.07, p<.001) were found. The significant preexposure age X preexposure condition interaction (F (2,46) = 5.93, p<.01) demonstrates the differential effect that context preexposure had on conditioning across the three age groups. Post-hoc Newman-Keuls tests reveal the PND 31 preexposed-same group (29.18 ± 7.59%) differed significantly from the PND 31 preexposed-other group (3.68 ± 0.82%) and all other groups (ps<.05), while no other groups differed (ps>.17) These results demonstrate that the facilitation of fear conditioning by preexposure to the shock context a day prior to delivery of a single immediate foot-shock is only observed for animals preexposed on PND 31. PND 17 animals exhibited remarkably low levels of fear conditioning to the context, regardless of preexposure condition (preexposed-same: 5.36 ± 1.21%; preexposed-other: 4.91 ± 1.02%) Experiment 1 then, suggests the CPFE-ISD develops later in ontogeny (between PND 24 and PND 31) when compared to conventional contextual fear conditioning, which is believed to emerge between PND 17 and PND 23 (Rudy & Morledge, 1994; Stanton, 2000). The lack of a significant difference (p>.20) between the PND 24 preexposed-same (14.93 ± 4.48%) and preexposed-other groups (8.13 ± 1.26%) is not consistent with previous research demonstrating a general CPFE around PND 24 (Rudy & Morledge, 1994) or a specific CPFE in the CPFE-ISD paradigm for animals conditioned at that same age (Burman et al., *under revision*). Perhaps the time between placement into the conditioning chamber and the delivery of the immediate shock was too short for the contextual representation to be retrieved for the PND 24 preexposed-same animals. In addition, a great deal of variation is expected at the approximate age (PND 23) of emergence for the contextual fear conditioning ability. The ongoing development of the hippocampus

and the hippocampus-dependent CAS results in variable degrees of CAS functioning between animals at that age. Specifically, the CAS for some animals has developed enough to support successful conditioning in the task, whereas other animals may not be sufficiently developed. As the CPFE-ISD paradigm is believed to selectively favor utilization of the CAS, this variable development may account for the lack of a CPFE observed for animals preexposed on PND 24. To investigate this account, various training parameters were manipulated in subsequent experiments to further examine the ontogeny of the context preexposure facilitation effect on contextual fear conditioning.

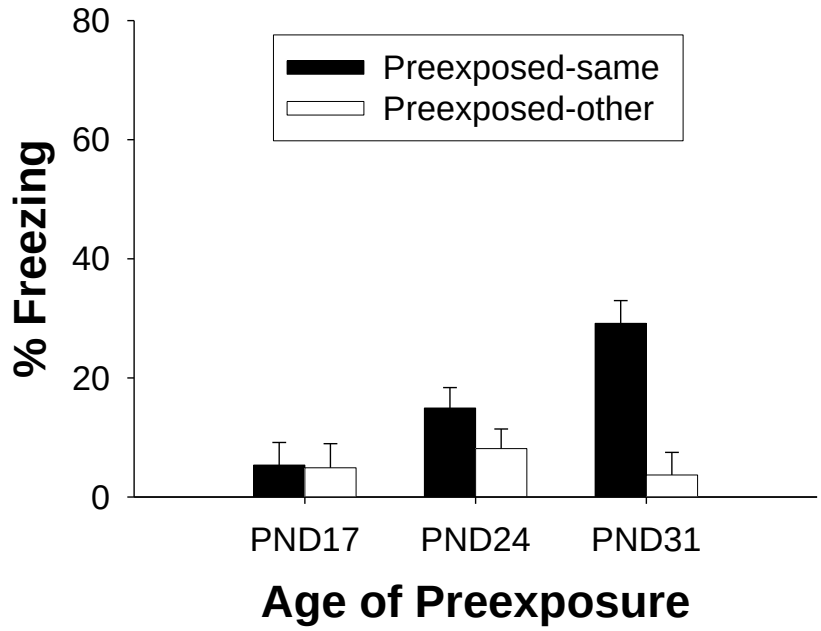

Figure 6. Ontogeny of the Context Preexposure Facilitation Effect on the Immediate Shock Deficit

Mean percent freezing displayed for preexposed-same (black) and preexposed-other (white) groups across ages of preexposure. Bars represent standard errors of the mean. CPFE-ISD was observed only for animals preexposed on PND 31.

Chapter 3

EXPERIMENT 2

Using the CPFE-ISD paradigm, Experiment 1 demonstrated a significant CPFE for animals preexposed on PND 31. However, a significant CPFE was not observed for animals preexposed on PND 24. Furthermore, generally low levels of contextual fear conditioning were observed for that age group regardless of preexposure condition. To test the variable development account, Experiment 2 examined the effect of various placement-to-shock intervals on conditioning levels across the three age groups in question. The design of Experiment 2A was a 2 (preexposure condition: preexposed-same vs. preexposed-other) x 3 (placement-to-shock interval: immediate vs. 10s vs. 30s) factorial for animals preexposed on PND 24. This design tested the hypothesis that, relative to immediate shock, the 10s or 30s PSI would give the preexposed-same animals enough time to retrieve its previously formed context memory, so that it could be associated with the shock. Any benefits of context preexposure would be demonstrated by a significant CPFE. To further develop the ontogenetic profile, Experiment 2B examined the effect of various PSIs on conditioning levels for both PND 17 and PND 31 preexposed animals. Since a significant CPFE was demonstrated for PND 31 preexposed animals in Experiment 1, a 2 (preexposure condition: preexposed-same vs. preexposed-other) x 3 (placement-to-shock interval: immediate vs. 30s vs. 120s) factorial design was used for the PND 31 animals. Significant CPFEs were expected for both the immediate and 30s PSI groups, while the 120s PSI would eliminate the CPFE, possibly due to a ceiling effect on

freezing levels. As low levels of conditioning were expected for all the PND 17 preexposed animals, a 2 (preexposure condition: preexposed-same vs. preexposed-other) x 2 (placement-to-shock interval: immediate vs. 120s) factorial design was used for the PND 17 animals. Even the longest PSI of 120s was expected to result in low levels of conditioning for the youngest rats, and no CPFEs were expected.

Another purpose of this experiment was to directly compare the general CPFE with conventional contextual conditioning established at the longer PSIs. The effect of PSI on standard contextual fear conditioning can be observed for animals preexposed to the other context: as the PSI increases, observed levels of fear conditioning should increase in those preexposed-other animals that can be conditioned to fear the context. Similar ontogenetic profiles between the CPFE and conventional contextual fear conditioning may suggest common underlying biological substrates/associative mechanisms that mediate the learning required for both variants of the conditioning task.

Experiment 2A

Methods

Subjects

A total of 61 Long Evans weanling rats (30 male, 31 female) that were the offspring of 10 different mothers were used in this study. Rats were bred, culled, reared, etc. as described previously (Experiment 1). No more than a single same-sex littermate was assigned to a given experimental group.

Apparatus and Stimuli

The apparatus and training context were the same as in Experiment 1.

Behavioral Procedure

Context preexposure (PND 24) and testing (PND 25) followed identical procedures as Experiment 1, except with two (rather than 4) animals being placed in their respective chambers at a time.

As in the training protocol for Experiment 1 (PND 25), two rats differing in preexposure condition were weighed, transferred into the transport cages and carted over to the training room. Unlike Experiment 1, only one rat was trained at a time to maximize precise timing of shock delivery, while the other rat remained in a room across the hall. Rats received a single foot-shock (1.5mA, 2 s duration) after various placement-to-shock intervals. The shock was delivered either immediately upon placement, following a 10 s delay or a 30 s delay. The immediate shock condition replicated Experiment 1 for PND 24 preexposed animals with the slight modification in training procedure. Load order was counterbalanced across preexposure condition, placement-to-shock interval and gender.

Data Analysis and Statistics

Data analysis and statistical tests were performed using the same programs as Experiment 1. Two males and 3 female rats were excluded following the statistical outlier analysis previously described. No more than a single rat was excluded per group. The rats that remained were distributed across the six groups as follows: 5 male and 4 female rats in the PND 24 preexposed-same immediate shock group, 6 male and 4 female rats in the PND 24 preexposed-other immediate shock group, 5 male and 4 female rats in the PND 24 preexposed-same 10 s PSI group, and 4 male and 5 female rats in the PND 24 preexposed-other 10 s PSI, 4 male and 5 female rats in the PND 24 preexposed-same 30 s PSI group, 5 male and 5 female rats in the PND

24 preexposed-other 30 s PSI group. Data were analyzed by means of a 2 x 2 x 3 factorial ANOVA (with sex, preexposure condition and placement-to-shock interval as independent factors). When usage of the Newman-Keuls Post-hoc analysis was not justified (if interactions were not significant), the originally designed orthogonal set of three planned comparisons investigated the potential CPFE within each PSI group by comparing the mean percent freezing levels between the different preexposure conditions.

Results & Discussion

The results from this experiment are displayed in Figure 7. There were significant main effects of gender (F (1,44) = 6.00, p<.05), preexposure condition (F (1,44) = 18.74, p<.001), and placement-to-shock interval (F (1,44) = 15.23, p<.001). The gender effect reflected the tendency of males (35.34 ± 5.06%) to freeze more than females (25.85 ± 4.09%). However, there were no significant interactions between gender and either of the other two variables of preexposure condition or placement-to-shock interval (*ps*>.16) so data are shown collapsed across gender. Furthermore, no significant interactions were found between preexposure condition and placement-to-shock interval or between all three variables (*ps*>.64).

The results of the three planned comparisons follow. Within the immediate shock group, the preexposed-same rats (25.49 ± 6.23%) froze at significantly higher levels than preexposed-other rats (4.42 ± 0.8%) (F (1,17) = 12.54, p<.01). This increase in freezing levels across preexposure condition was also observed in the 10 s delay groups, with preexposed-same rats (37.92 ± 4.17) freezing more than preexposed-other rats (18.22 ± 4.4)(F (1,16) = 10.56, p<.01). However, no significant difference was found between the freezing levels of preexposed-same

(59.98 ± 5.93%) and preexposed-other (43.95 ± 10.02) rats who were shocked after a 30 s delay (F (1,17) = 0.84, p>.373)

The main effect of preexposure condition and PSI, taken together with analysis of the planned comparisons, reveal an interesting pattern of results. Even though context preexposure generally enhances conditioning, the CPFE is only observed for the immediate and 10 s delay shock groups. The preexposed-other immediate PSI group demonstrated the ISD, and froze very little throughout the testing session. Enhanced levels of freezing for the preexposed-same immediate PSI group indicated the CPFE-ISD had occurred. The significant CPFE-ISD for these PND 24 preexposed animals contrasts with the results of Experiment 1, where no CPFE-ISD was found. Stronger transport cue-preexposed context associations for Experiment 2A preexposed-same animals cannot adequately explain the difference as both the transport cues and general preexposure procedure were held constant across experiments. However, the new found CPFE-ISD observation may be consistent with the variable development account of contextual fear conditioning at this transitional age (PND24). Furthermore, loading one animal at a time during training may have ensured a more immediate delivery of a shock. A potentially greater ISD could have resulted as the preexposed-other animals could not benefit from the additional ~2 seconds of context exposure before the shock as for those loaded first in Experiment 1.

The main effect of placement-to-shock interval and the directional pattern of change in freezing levels across PSIs demonstrate that conditioning is enhanced as the PSI increases for both preexposure conditions. The conventional contextual fear conditioning ability has clearly developed by PND 25, evidenced by the high levels of fear being expressed by the 30s PSI preexposed-other group who were trained at that

age. Clearly, the time before the shock plays an integral role in the amount of conditioning one can expect in the preexposed-other groups. If conventional contextual fear conditioning truly does depend upon the formation of the contextual conjunctive representation, then it seems 30 s is long enough for the PND 25 trained animal to construct the context memory and associate it with the shock. In fact, it seems context preexposure loses it facilitating effect when the long PSI is used, so that both the preexposed-same and preexposed-other animals freeze at similar levels. However, the CPFE may be masked by a ceiling effect generated from the specific parameters used to establish and assess conditioning (consider the general CPFE reported after a 120s PSI and 10 min retention interval for PND 23 trained animals in Rudy & Morledge (1994)). Ceiling levels following a 30s PSI and apparent elimination of the CPFE is consistent with the reports from Burman et al., (*under revision*), where PND 23 preexposed animals froze at similarly high levels across preexposure conditions and between the 30s and 120s PSIs. Minor levels of freezing are reported for the preexposed-other 10s PSI group, suggesting some sort of (CAS or SAS-mediated) conditioning may have taken place. That being said, the preexposed-same animals in the 10s PSI group still demonstrated enhanced fear conditioning, evidenced by the significant CPFE. In general, the CPFE is presumed to be accounted for by the *conjunctive view*, but Pugh & Rudy (1996) reported support for the *enhanced saliency view* for animals conditioned at similar ages. This experiment did not perform a feature vs. context preexposure manipulation and so the *exact* nature of the CPFEs for both the ISD and the 10s PSI cannot be determined for PND 24 preexposed animals, even though the SAS is not believed to be able to support conditioning in the CPFE-ISD paradigm. However, both the CPFE (of the ISD and the

more general) and the conventional contextual fear conditioning ability were observed for PND 24 animals, in contrast to the results from Experiment 1.

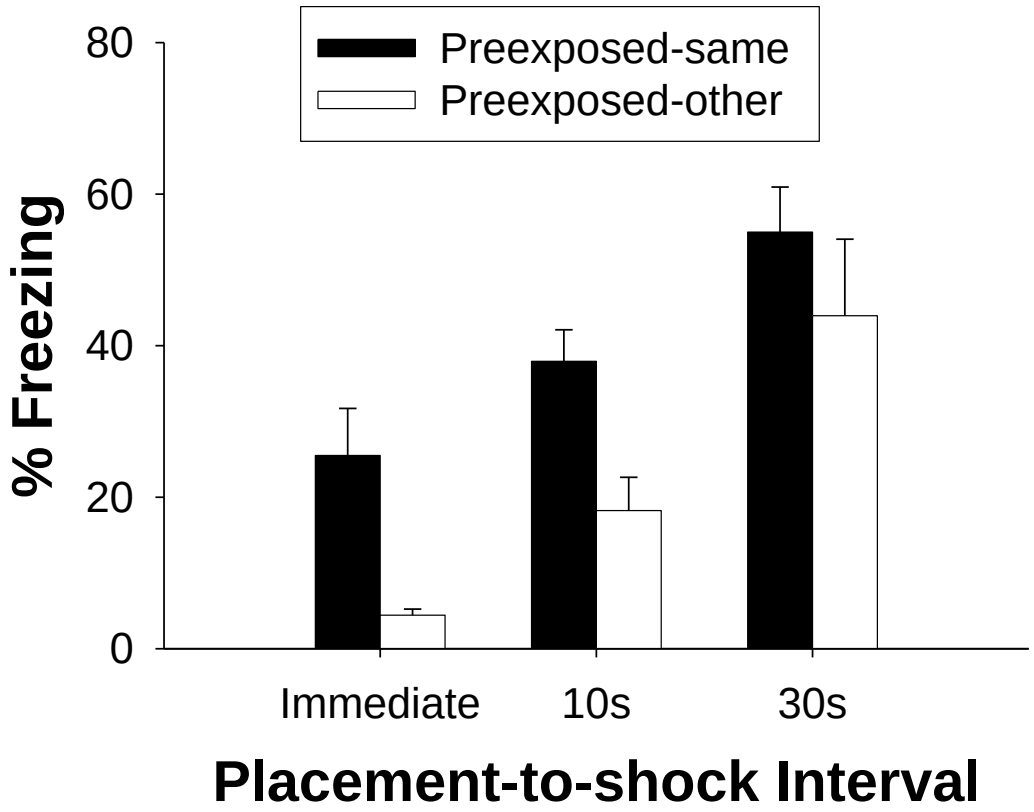

Figure 7. Percent Freezing Across Various Placement-to-shock Intervals for Rats Preexposed on PND 24

Mean percent freezing displayed for preexposed-same (black) and preexposed-other (white) groups. Bars represent standard errors of the mean. On the training day, the shock was delivered after various placement-to-shock intervals. CPFE observed for both immediate and 10s placement-to-shock interval groups, but not for the 30s group.

Experiment 2B

Methods

Subjects

A total of 115 Long Evans PND17 or 31 rats (57 male, 58 female) that were the offspring of 23 different mothers were used in this study. Rats were bred, culled, reared, etc. as described previously (Experiment 1). No more than a single same-sex littermate was assigned to a given experimental group.

Apparatus and Stimuli

The apparatus and training context were the same as in Experiment 1.

Behavioral Procedure

Context preexposure (PND 17 or 31) and testing (PND 19 or 33) followed identical procedures as Experiment 2A.

Training (PND 18 or 32) was the same as Experiment 2A except different placement-to-shock intervals were used. PND 17 preexposed animals received the foot-shock either immediately upon placement or after a 120 s delay. PND 31 preexposed animals had the shock administered either immediately, following a 30 s delay or a 120 s delay. Animals were transported to the training chambers in pairs matched for age while differing in preexposure condition. Once again, load order was counterbalanced across preexposure condition, placement-to-shock interval and gender.

Data Analysis and Statistics

Data analysis and statistical tests were as described previously. Two males and 3 female rats were excluded as statistical outliers. The rats that remained were distributed as follows (with number of outliers removed in parentheses, if any). For the PND 17 preexposed-same groups: 6 male and 5 female rats in each of the immediate shock and 120s PSI (1) groups. For the PND 17 preexposed-other groups: 5 male and 6 female rats in each of the immediate shock and 120s PSI (1) groups. For the PND 31 preexposed-same groups: 7 male and 4 female rats to the immediate shock (2) group, 5 male and 6 female rats to the 30 s PSI group, and 5 male and 5 female rats to the 120 s PSI group. For the PND 31 preexposed-other groups: 6 male and 6 female rats to the immediate shock group (1), 6 male and 5 female to the 30 PSI group, and 4 male and 6 female rats to 120 s PSI group. A 2 x 2 x 2 factorial ANOVA was run (with sex, preexposure condition and placement-to-shock interval as independent factors) on the data from the PND 17 preexposed animals and a separate 2 x 2 x 3 factorial ANOVA was run (with sex, preexposure condition and placement-to-shock interval as independent factors) on the data from the PND 31 preexposed animals. Newman-Keuls post-hoc tests were used to further examine trends found in the ANOVA.

Results & Discussion

The results of the analysis of the data from the PND 17 preexposed animals appear in Figure 8. There was no effect of gender (F (1,37) = 0.42, p>0.52), nor any interactions with gender (ps>.24). There was no main effect of preexposure (F (1,37) = 1.84, p>.18) which indicates preexposure did not generally enhance conditioning. There was a trend towards a significant main effect of PSI (F (1,37) = 3.01, p<.10), with animals trained in the immediate shock condition potentially

demonstrating lower levels of conditioning than those trained with the 120 s PSI. There was no preexposure x PSI interaction (F (1,37) = 0.55, p>.46), as the effect of preexposure did not differ between the two the placement-to-shock conditions. Despite the trend towards a significant main effect of placement-to-shock interval, the most notable finding from analysis of the PND 17 preexposed animal data is the continued low levels of conditioning when compared to those observed for older rats (see Experiment 2A and PND 31 data from this experiment). As rats at this age can demonstrate post-shock freezing and explicit cue conditioning, the inability for rats preexposed on PND 17 to express fear (performance account) cannot be the reason for the observed differences. Furthermore, Pugh & Rudy (1996) demonstrated that rats conditioned at a comparable age could exhibit some level of contextual fear conditioning. Although, the conditioning could only be established in a "salient" black context and the reported CPFE promoted the *enhanced saliency view*; two findings that suggest the conditioning was SAS-mediated. The potential facilitation of SAS-mediated conditioning likely did not occur in this experiment since a clear context was used as opposed to a "salient" black one. Therefore, the consistently low levels of expressed fear reported for PND 17 preexposed animals in these experiments is probably due to deficits involved in the formation of the conjunctive contextual representation, associating the memory with the shock stimulus, or retrieval of the context-shock association when tested. The latter two alternatives seem unlikely, as contextual fear conditioning (along with auditory-cue fear conditioning) has been reported for animals around this age (Pugh & Rudy, 1996), generally suggesting the rats have functioning associative/retrieval memory mechanisms.

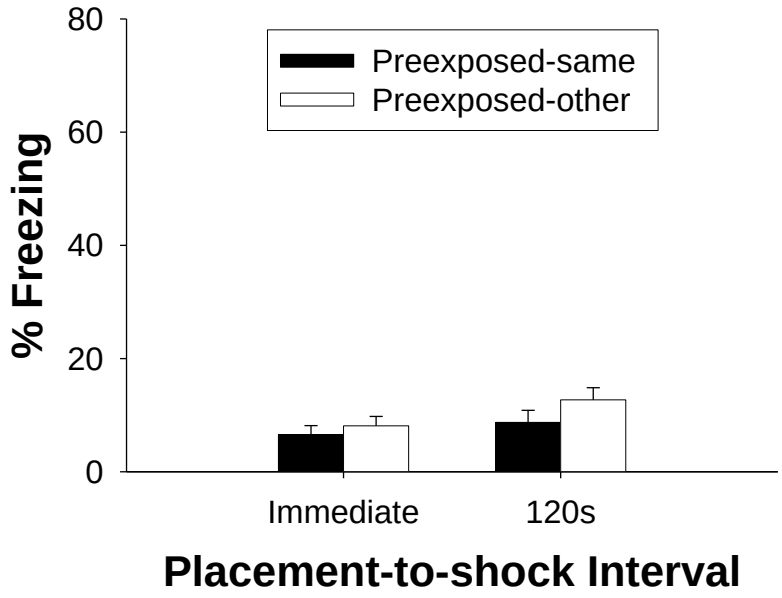

Figure 8. Percent Freezing for Animals Preexposed on PND 17 and Trained with Immediate vs. 120s Placement-to-shock Intervals

Mean percent freezing displayed for preexposed-same (black) and preexposed-other (white) groups. Bars represent standard errors of the mean. The shock was administered after either an Immediate or 120s placement-to-shock interval. No CPFE was observed, and conditioning levels were low regardless of preexposure condition or placement-to-shock interval, suggesting PND 17 preexposed animals cannot support conventional contextual fear conditioning or conditioning in the CPFE-ISD paradigm.

The PND 31 preexposed results are depicted in Figure 9. There was no main effect of gender (F (1,53) = 0.01, p>.92), nor an interaction between gender and placement-to-shock interval (p>.94). There was a trend towards an interaction between gender and preexposure condition (F (1,53) = 2.88, p<.10), representing the slight trend towards a greater effect of preexposure in female rats. There was also a trend towards a significant main effect of preexposure (F (1,53) = 3.77, p<.06). A significant

main effect of placement-to-shock interval was observed (F (2,53) = 42.19, p<.001), characterized by lower levels of conditioning in the immediate shock group. Most importantly, there was a significant preexposure x PSI interaction (F (2,53) = 4.94, p<.05), where the CPFE was only observed for the immediate shock group. Indeed, posthoc Newman-Keuls tests show that both the preexposed-same (27.2 ± 4.43%) and preexposed-other (7.61 ± 1.94%) immediate shock groups differed significantly from each other and every other group (ps<.01), whereas no others groups significantly differed (ps>.14). As in Experiment 2A for animals preexposed on PND 24, the CPFE is only observed if the shock is delivered immediately upon placement, and it is abolished if placement and shock administration are separated by 30s. Furthermore, freezing levels may have reached ceiling values in the 30s PSI group (61.10 ± 4.44% collapsed across preexposure condition) as similar conditioning levels are observed for animals trained with a 120s PSI (61.62 ± 4.33% collapsed across preexposure condition). The effect of increasing PSIs seems to produce a similar pattern of results for animals preexposed on PND 24 (Experiment 2A) and PND 31. The observed ISD and robust conditioning following the 30s PSI for PND 31 preexposed-other animals were comparable to the results for PND 24 preexposed-other animals in Experiment 2A; rats that demonstrated a similar increase in freezing from low levels to ceiling levels across the same PSI window.

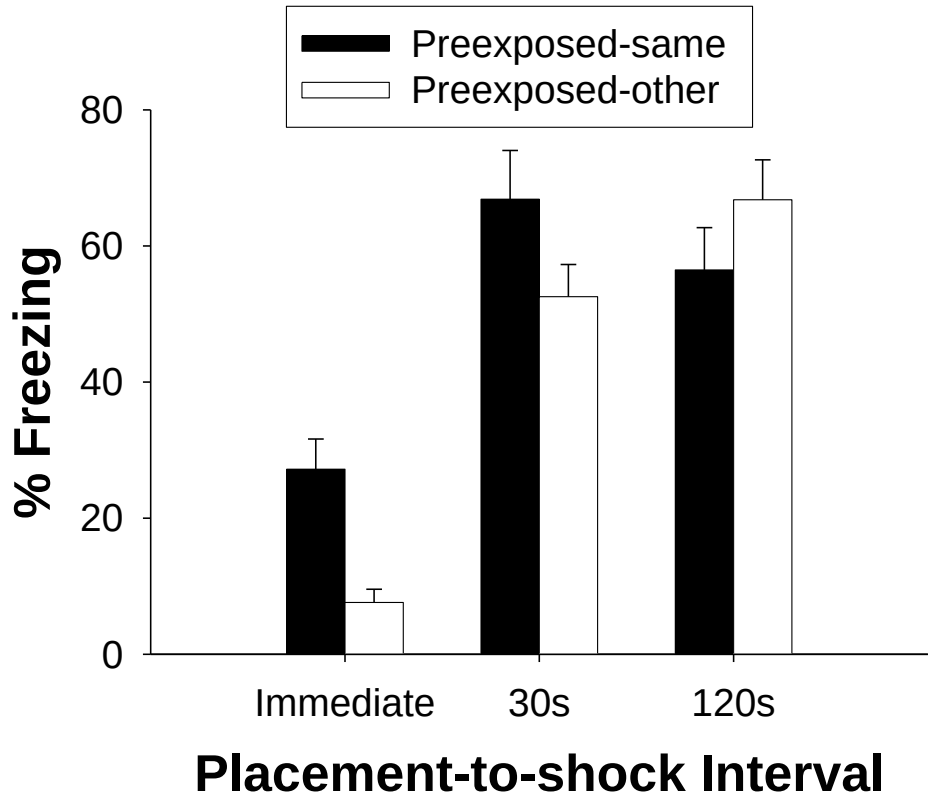

Figure 9. Percent Freezing for Animals Preexposed on PND 31 and Trained with Various Placement-to-shock Intervals

Mean percent freezing displayed for preexposed-same (black) and preexposed-other (white) groups. Bars represent standard errors of the mean. The shock was administered after various placement-to-shock intervals (Immediate, 30s, or 120s). CPFE observed only for immediate shock group, and similar levels of fear are expressed across the preexposure conditions of the two higher placement-to-shock intervals.

Further characterization of the ontogenetic profile for both the general CPFE and the conventional contextual fear conditioning ability is afforded by combining the implications of both Experiment 2A and 2B. PND 17 preexposed animals continued to exhibit markedly low levels of fear conditioning, even after the longest PSI. Focusing on preexposed-other animals, conventional contextual fear

conditioning emerged as a function of PSI, so that longer intervals produced higher levels of conditioning to a point. Both PND 24 and 31 preexposed-other animals demonstrated that relationship, and froze at comparable ceiling levels following a 30s PSI. Therefore, the conventional contextual fear conditioning ability rapidly emerges between PND 17 and PND 24 after which it seems to be functioning at mature levels (at least when compared to PND 31 animals). This developmental pattern supports PND 23 as the approximate age of emergence for the contextual fear conditioning as has been argued (Rudy & Morledge, 1994; Rudy, 1993; Stanton, 2000). The observation of a general CPFE for PND 24 & 31 preexposed animals (and lack of a general CPFE for PND 17 preexposed animals) is consistent with previous reports demonstrating a similar pattern of general CPFEs at comparable ages (Rudy & Morledge, 1994; Rudy & O'Reilly, 1999). Therefore, the general CPFE seems to emerge between PND 17 & PND 24, in parallel to the conventional contextual fear conditioning ability. In contrast to Experiment 1, the results from Experiment 2A and 2B collectively establish the CPFE-ISD, the CAS-dependent variant of the CPFE paradigm, emerges between PND 17 and PND 24, just as the general CPFE and conventional contextual fear conditioning abilities do. The parallel ontogenetic profiles of the three phenomena suggest common underlying biological substrates mediate a common associative strategy, both of which may develop to functional levels by PND 24. That the CPFE-ISD was seen on PND24 in Experiment 2 but not Experiment 1 likely reflects variable development together with the fact that the CPFE-ISD paradigm seems to be slightly more difficult task. The next experiment tested this idea further by manipulating additional training variables in an effort to better characterize the "parametric space" for the ontogeny of the CPFE-ISD.

Chapter 4

EXPERIMENT 3

The variation in the CPFE-ISD on PND24 observed across Experiments 1 and 2 (where training parameters were held relatively constant) could merely reflect weak training parameters. Understanding the optimal parameters at PND24 is potentially important for subsequent investigations into the neural substrates that mediate conditioning in the CAS-dependent task at ages when this process first emerges ontogenetically. To address this issue, Experiment 3 trained animals with two shocks, in hopes of increasing the strength of the context-shock association so that a more reliable CPFE-ISD could be observed. Furthermore, although training with a 10s PSI produced a significant CPFE in Experiment 2, elevated levels of freezing for the preexposed-other group were also observed. To be confident in the sole utilization of the CAS for the preexposed-same animals, low levels of freezing must be observed for the preexposed-other group. Therefore, Experiment 3 trained animals with an immediate PSI, which consistently resulted in low levels of expressed fear in preexposed-other animals across Experiments 1 and 2, regardless of age. Multiple shocks during training were also given to animals preexposed on PND 17 and PND 31 to address the role of this variable in ontogenetic comparisons. Thereby, a 2 (preexposure condition: preexposed-same vs. preexposed-other) x 3 (age of preexposure: PND 17 vs. PND 24 vs. PND 31) factorial design was used to examine the effect of two shocks on the developmental profile. The PND 17 animals were expected to demonstrate low levels of conditioning in a manner consistent with the

results from previous experiments. Meanwhile, a significant CPFE-ISD was predicted for both the PND 24 and 31 preexposed groups. An additional 2 (preexposure condition: preexposed-same vs. preexposed-other) x 2 (number of shocks: 1 vs. 2) factorial design for PND 24 preexposed animals investigated the potential enhancement of conditioning resulting from the increased number of shocks. The single shock group also functioned to replicate the CPFE-ISD protocol used for PND 24 preexposed animals in previous experiments. In summary, Experiment 3 asked whether the increased number of shocks would affect the CPFE-ISD in contextual fear conditioning differentially across the developmental profile, and whether an additional shock during training would enhance conditioning levels at the approximate age of emergence (PND 24).

Methods

Subjects

A total of 87 Long Evans weanling rats (46 male, 41 female) that were the offspring of 21 different mothers were used in this study. Rats were bred, culled, reared, etc. as described previously (Experiment 1). No more than a single same-sex littermate was assigned to a given experimental group.

Apparatus and Stimuli

The apparatus and training context were the same as in Experiment 1.

Behavioral Procedure

Context preexposure (PND 17, 24, or 31) and testing (PND 19, 25, or 33) followed identical procedures as Experiment 2, with two animals run at a time.

For training (PND 18, 25, or 32), two rats matched for age while differing in preexposure condition were weighed, transferred into the transport cages and carted over to the training room. All of the PND 18 and 32 rats received two foot-shocks (separated by 1 s) immediately after placement (approximately 3 s placement-to-shock interval). Load order was counterbalanced across preexposure condition and gender. PND 25 rats were trained with either a single foot-shock (quasi-replicating Experiment 1 & immediate group from Experiment 2A) or two foot-shocks as just described. Again, rats were removed from the chambers as quickly as possible following the foot-shock, returned to their housing and left for approximately 24 hours until testing.

Data Analysis and Statistics

Data analysis and statistical tests were performed using the same programs as previous experiments. Four males and 4 female rats were excluded as statistical outliers, one from each group. The rats that remained were distributed across the eight groups as follows: 3 male and 4 female rats in the PND 17 preexposed-same group, 4 male and 3 female rats in the PND 17 preexposed-other group, 4 male and 5 female rats in the PND 24 single shock preexposed-same group, 2 male and 7 female rats in the PND 24 single shock preexposed-other group, 5 male and 3 female rats in the PND 24 two shock preexposed-same group, 4 male and 4 female rats in the PND 24 two shock preexposed-other group, 9 male and 8 female rats in the PND 31 preexposed-same group, and 7 male and 7 female rats in the PND 31 preexposed-other group. A 2 x 2 x 3 factorial ANOVA was run (with sex, preexposure condition and age of preexposure as independent factors) on the data collected from animals trained with two shocks. An additional 2 x 2 x 2 factorial ANOVA was performed (with sex, preexposure condition and number of shocks as independent factors) on the data

collected from PND 24 preexposed animals. When valid, Newman-Keuls post-hoc analysis was used to further examine trends found in the ANOVAs. Otherwise, an orthogonal pair of planned comparisons evaluated potential freezing level differences between the preexposure conditions given a single shock to analyze the potential CPFE, or between the number of shocks within the preexposed-same group to investigate the potential enhancement of conditioning following an additional shock for PND 24 preexposed-same animals.

Results & Discussion

The results from the analysis of the multiple shock data across the three ages are displayed in Figure 10. There was no effect of gender (F (1,49) = .001, p>.96) nor were there any interactions with gender (ps>.49). Significant main effects of preexposure age (F (2,49) = 3.57, p<.05) and preexposure condition (F (1,49) = 15.83, p<.001) were found. A significant age x preexposure condition interaction (F (2,49) = 3.31, p<.05) again revealed the differential effect context preexposure had on conditioning across the three ages. Newman-Keuls post-hoc analysis reveal both the PND 24 (30.63 ± 5.61%) and PND 31 (29.75 ± 4.90%) preexposed-same groups differed from all other groups (ps<.01) except each other, while none of the other groups differed (ps>.86). Therefore, when training consists of two shocks immediately delivered upon placement in the conditioning chamber, significant CPFEs were observed for both PND 24 and PND 31 animals. These results differ from Experiment 1, which did not find a significant CPFE-ISD for animals preexposed on PND 24, but are consistent with the results from Experiment 2A, in which a significant CPFE-ISD was observed. Interestingly, multiple shocks resulted in the PND 24 preexposed-same group displaying similar levels of conditioning as the PND 31 preexposed-same group,

where they had exhibited significantly lower levels of fear when the same groups were compared in Experiment 1. The significant CPFE-ISD for PND 31 preexposed animals given two shocks demonstrates the reliability of the CPFE-ISD at that age. Furthermore, the second shock didn't seem to enhance conditioning for the PND 31 preexposed-same animals across experiments (as it had for the PND 24 preexposed-same animals), as similar levels of freezing were reported in Experiments 1 and 2B after a single immediate shock was delivered. Consistent cross-experiment results were also reported for PND 17 preexposed animals, which continued to demonstrate an absence of fear conditioning to the context even after two-shock training.

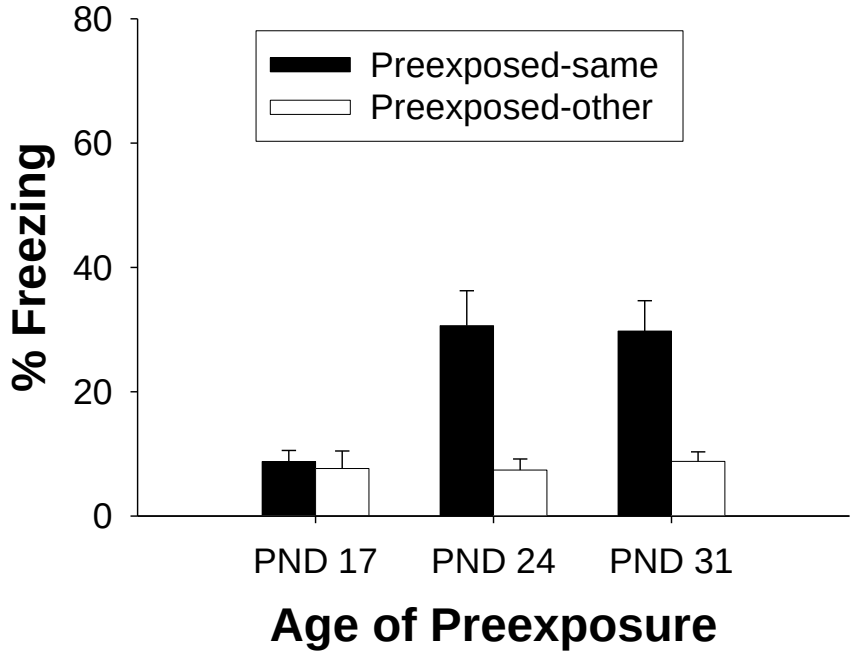

Figure 10. Effect of Two Shocks on Developmental Profile of CPFE-ISD

Mean percent freezing displayed for preexposed-same (black) and preexposed-other (white) groups across ages of preexposure (PND 17, 24, and 31). Bars represent standard errors of the mean. Two shocks were administered immediately upon placement. CPFE-ISD was observed for both PND 24 and PND 31 preexposed-same groups, and unlike Experiment 1, the freezing values did not differ.

The results from the comparison of PND 24 single shock training versus PND 24 two-shock training across preexposure condition are illustrated in Figure 11. Following ANOVA, there was no effect of gender (F (1,26) = .61, p>.44) or number of shocks (F (1,26) = 2.18, p>.15), nor were there interactions between any of the variables (ps>.15). A significant main effect of preexposure condition (F (1,26) = 19.05, p<.001) indicates a general enhancement of conditioning for animals preexposed to the shock context.

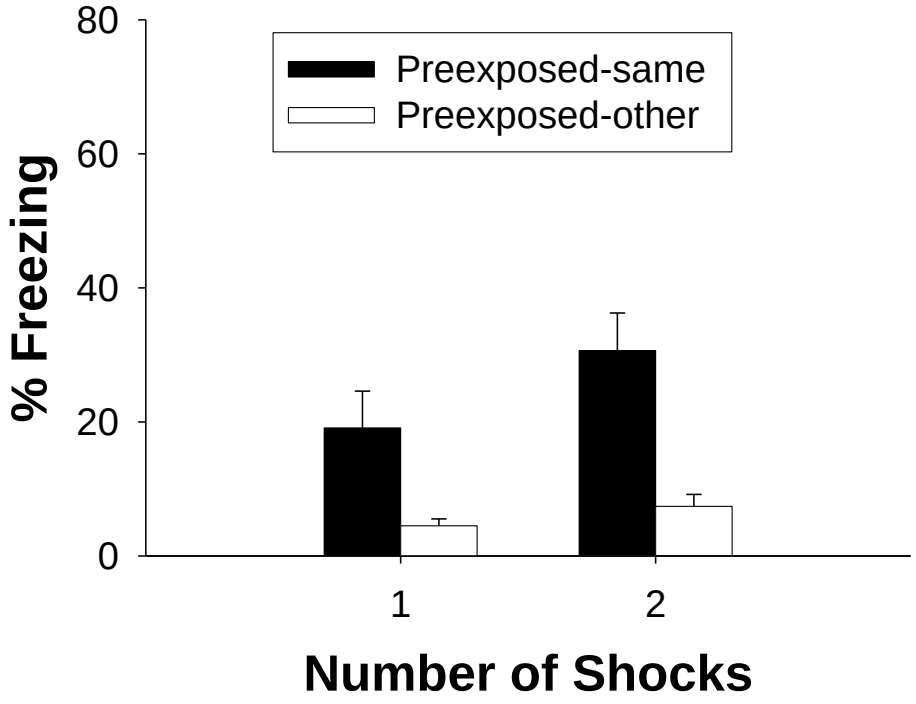

Figure 11. Effects of Training with One vs. Two Immediately Delivered Shocks on Freezing Levels for PND 24 Preexposed Animals

Mean percent freezing displayed for preexposed-same (black) and preexposed-other (white) groups across ages of preexposure (bars represent ± SEM). PND 24 data from Experiment 2 compared to single shock quasi-replication of group PND 24 preexposed group from Experiment 1 with animals loaded one at a time. CPFE-ISD observed for both shock groups. Conditioning for preexposed-same animals may have been enhanced by additional shock, but the difference was not supported statistically.

For the PND 24 single shock group planned comparison, the preexposed-same (19.09 ± 5.49%) group differed significantly from the preexposed-other (4.49 ± 1.03%) group (F (1,16) = 6.83, p<.05), with the preexposed-same group exhibiting higher levels of freezing. These results of this quasi-replication differ from those in Experiment 1 which did not find a significant CPFE-ISD for PND 24 preexposed animals receiving a single shock during training. Meanwhile, the results replicate the significant CPFE-ISD observed in Experiment 2A. Once again, the variable development account may explain why a significant CPFE-ISD is found in this experiment and Experiment 2A but not in Experiment 1, when nearly identical training parameters were used across experiments. The comparison of preexposed-same groups across one shock (19.09 ± 5.49%) and two shocks (30.63 ± 5.61%) did not result in a significant difference (F (1,15) = 2.15, p>.16), so a significant multiple shock enhancement for the preexposed-same animals was not statistically supported.

Collectively, the results from this experiment suggest training with an additional shock had variable effects on the CPFE-ISD and contextual fear conditioning across ontogeny. Specifically, the patterns of conditioning for PND 17 and 31 preexposed animals did not seem to change in response to the additional shock when comparing the data with those collected from immediate single shock training in Experiments 1 and 2B. The post-hoc analysis of the ontogenetic multiple shock data revealed the PND 24 preexposed-same animals froze at similar levels to the PND 31 preexposed-same animals. This finding was in direct contrast with the results of Experiment 1, which reported the PND 31 preexposed-same animals froze at significantly higher levels. However, comparing the mean freezing levels for the PND 24 (25.49 ± 6.23%) and 31 (27.2 ± 4.43%) preexposed-same, immediate single shock

animals from Experiment 2A and 2B reveals the two groups may freeze at identical levels under those training conditions. In this experiment, the mean freezing levels for the PND 24 preexposed-same animals given a single shock (19.09 ± 5.49%) seemed to be lower than the conditioning levels reported for the two shock group (30.63 ± 5.61%), even though the difference was not statistically significant. Applying the variable development account to the CPFE-ISD, it would seem that (based on average) the PND 24 preexposed-same animals from Experiment 1 were underdeveloped, those from Experiment 2A had developed to levels functionally equivalent to older rats, and the development for those from this experiment was at some intermediate level. Furthermore, the additional shock had no effect on conditioning patterns for the PND 17 preexposed animals as they were too underdeveloped to support any levels of conditioning in the CPFE-ISD paradigm. Conversely, the additional shock did not seem to enhance conditioning levels for PND 31 preexposed-same animals as they may have already developed enough to be able to form a strong enough context-shock association that can effectively, reliably, and sufficiently control behavior enough to exhibit a CPFE-ISD after just a single shock.. Even though a significant enhancement of conditioning following the additional shock for the PND 24 preexposed-same animals was not statistically significant, the observation of a significant CPFE at a lower alpha level for the two-shock trained group ($p<.01$) than that of the single shock group ($p<.05$) along with seemingly greater average freezing values encouraged the implementation of a multiple shock training procedure for future experiments.

Chapter 5

EXPERIMENT 4

The purpose of Experiment 4 was to determine the role of hippocampal NMDA receptors in the CPFE-ISD in weanling rats. As described previously (see General Introduction), the hippocampus has been shown to play a critical role across the CPFE paradigm of contextual fear conditioning for adult rats during (1) the formation of the conjunctive contextual representation during the preexposure phase, (2) the transient storage and activation of the conjunctive memory so that it may be associated with the shock during the training phase, and (3) the retrieval of the conjunctive memory during the test phase so that the context-shock association can be activated to subsequently generate the observed behavioral fear response (Matus-Amat, 2004). The mnemonic function (1) is believed to rely on NMDA-receptor dependent plasticity via long term potentiation (LTP) that occurs within the neurons of the hippocampus, particularly the dorsal region (Young et al., 1994; Maren, 2001; Stote & Fanselow, 2004; Fanselow & Poulos, 2005). Antagonism of NMDA-receptors by infusing MK-801 into the dorsal hippocampus of weanling rats has been shown to impair other spatial learning tasks, such as delayed alternation (Watson et al., 2009) Based upon these findings, this experiment examined the effect of NMDA-receptor antagonism in the DH of weanling rats during the preexposure phase of the CPFE-ISD paradigm, when the hippocampus is believed to perform its configural mnemonic function. Although it is generally believed that contextual fear conditioning ability and general CPFE is mediated by the hippocampus throughout the rat's life, the role of the

hippocampus has never been investigated at the approximate age of emergence of the CPFE (PND 24). Pugh & Rudy (1996) demonstrate the critical importance of this, as their results supported the *enhanced saliency view* of the CPFE for similarly aged rats. The ongoing development of the hippocampus and the hippocampal-amygdala/cortical interactions across ontogeny suggest that certain functions of hippocampal-dependent CAS may emerge before others. The results from Pugh & Rudy (1996) may be due to this dissociated development of function. Although, contextual fear conditioning is generally believed to be mediated by the hippocampal-dependent CAS in intact rats, Pugh & Rudy (1996) report the apparent utilization of the hippocampal-independent SAS in the CPFE observed for PND 18 and PND 23 trained rats. As PND 23 is consistently argued to be the approximate age of emergence for hippocampus-dependent contextual fear conditioning (Rudy, 1993; Rudy & Morledge, 1994; Stanton, 2000), the apparent utilization of the SAS reported in Pugh & Rudy (1996) for PND 23 animals trained with a 120s PSI may suggest the SAS-inhibiting function of the hippocampal-dependent CAS emerges a little later in ontogeny relative to its mnemonic function. The potential confounding hippocampal-independent SAS contributions to conditioning raises uncertainty regarding the mnemonic role of the hippocampus in contextual fear conditioning early in ontogeny. Therefore, it becomes critical to employ the CPFE-ISD paradigm because it minimizes the potentially confounding role of SAS and selectively requires utilization of the hippocampal-dependent CAS. Burman et al., (*under revision*) began the investigation into the neuromolecular determinants of the CPFE-ISD at the age of emergence by systemically administering the NMDA-type glutamate receptor antagonist, MK-801, before the preexposure phase. The observed deficit in conditioning for animals

receiving the drug relative to the saline control group during the preexposure phase implicated a general role for NMDA-receptors in the formation of the conjunctive representation. However, systemic administration of MK-801 targets numerous brain regions, so the NMDA-dependent contribution of particular neural structures cannot be determined. To address this issue, this experiment infused MK-801 bilaterally into the DH before the preexposure phase to examine the role of NMDA receptors in the dorsal hippocampus in mediating the formation of the contextual representation. We predicted that antagonism of hippocampal NMDA receptors during preexposure would impair the CPFE-ISD in PND24 rats, as it does in adult rats (Young et al., 1994; Stote & Fanselow, 2004).

A 2 (preexposure condition: preexposed-same vs. preexposed-other) x 3 (drug treatment: MK-801 vs. saline vs. un-operated) factorial design was used. The un-operated groups were included to determine whether surgical installation of cannulas alters the CPFE-ISD. The training parameters that seemed to maximize the CPFE-ISD for PND 24 preexposed animals in preceding experiments, including delivery of 2 shocks (Experiment 3), were used. A significant CPFE-ISD was anticipated when comparing freezing values across preexposed-same vs. preexposed other in the control groups. MK-801 infusion into the DH before preexposure was expected to disrupt the NMDA-dependent formation of the conjunctive representation that normally would occur during preexposure. Therefore, no increase in freezing was predicted in the MK-801 preexposed-same group relative to the MK-801 preexposed-other group.

Methods

Subjects

A total of 45 Long Evans weanling rats (22 male, 23 female) that were the offspring of 11 different mothers were used in this study. Rats were bred, culled, reared, etc. as described previously (Experiment 1). No more than a single same-sex littermate was assigned to a given experimental group, except a single inadvertent case in which the behavioral data of the two subjects were averaged and included in the statistical analysis as a single observation.

Surgery

Cannulation surgeries were performed as described in Watson et al. (2009). Rats were taken individually from post-weaning group housing on PND 22 and anesthetized with and ketamine/xylazine mixture (52.2-60.9 mg/kg ketamine/7.8-9.1 mg/kg xylazine in a 1.0 ml/kg injection volume) prior to surgery. Once anesthetized, animals were prepared for surgery and placed into a stereotaxic frame apparatus. Stainless-steel guide cannulas (Plastics One, Roanoke, VA) were implanted bilaterally to terminate in the dorsal hippocampus (DH) according to the coordinates and procedure of Watson et al. (2009). The following coordinates were used for bilateral DH implantation: anteroposterior (AP), +2.6 mm relative to interaural midline; mediolateral (ML), ± 2.3 mm and dorsoventral (DV), -2.0 mm relative to bregma. Cannulas were fixed in place using dental acrylic on two "skull hooks" (Stanton & Freeman, 1994; Watson et al., 2009). Dummy cannulas were inserted into the guide cannulas following surgery to prevent obstruction until infusions were made. After surgery, rats were allowed to recover from anesthesia in individual white plastic cages (see Experiment 1: Apparatus for dimensions) with half the floor placed on

electric heating pads (Watson et al., 2009). Rats were given a full day (PND 23) to recover from surgery before the preexposure phase of the behavioral protocol began on PND 24. Un-operated control rats were weighed and transferred to individual cages on PND 22 like the operated rats but were left undisturbed until the start of the experiment.

Apparatus and Stimuli

The apparatus and training context were the same as in Experiment 1.

Drug Infusion

On PND 24 (preexposure phase), rats received microinjections of either MK-801 or sterile saline. Un-operated rats were handled in a similar manner and for an approximately equivalent duration as those rats receiving infusions. The preexposure phase began on average 32 minutes (± 3 min) after the infusion procedure.

The infusion procedure is based on that of Watson et al (2009). It began with the removal of the dummy cannulas, so that a microinjector cannula could be inserted into each guide cannula. The tip of the injector cannulas extended 1 mm below the end of the guide cannulas and into the DH. The injector cannulas were attached to 10 µL Hamilton syringes mounted on a microinfusion pump via polyethylene tubing. MK-801 (10 µg/ µL dissolved in sterile saline) was bilaterally infused at a rate of 0.25 µL per minute for a single minute. Therefore, rats received infusions of 0.25 µL, which equates to 2.5 µg of MK-801 in each side. This infusion volume has been shown not to spread beyond the DH in a separate experiment performed in the lab that utilized radioactive-labeled MK-801 (Burman, Rosen &

Stanton, *unpublished observations*). Saline controls received equivalent volumes of sterile saline at an identical rate. Between 2-4 animals were infused at a time and were allowed to roam freely in their individual home cages during administration. Infusion groups were counterbalanced by drug treatment, so that each group had at least one MK-801 infusion and one saline infusion. The injector cannulas were removed one minute after the completion of the infusion, and the dummy cannulas were reinserted into the guide cannulas. Animals remained in their home cages until the start of behavioral procedures. Once again, un-operated control rats were handled in a similar manner for a similar duration.

Behavioral Procedure

Context preexposure (PND 24) began between 15-45 minutes after the conclusion of the infusion procedure. Preexposure, training (PND 25) and testing (PND 26) followed identical behavioral procedures as two-shock PND 24 group in Experiment 3. During training, all rats received two foot-shocks (separated by 1 s) immediately after placement (approximately 3 s placement-to-shock delay). Two shocks were administered due to the results of the one versus two shock comparison for PND 24 preexposed animals in Experiment 3. Load order was counterbalanced across drug treatment condition and gender. Again, rats were removed from the chambers as quickly as possible following the foot-shock, returned to their home cage and left for approximately 24 hours until testing.

Histology

Within 24-48 hours after completion of behavioral testing, rats received a lethal overdose of the ketamine/xylazine mixture and were perfused under deep

anesthesia. Animals were perfused intracardially with 0.9% saline for two minutes followed by perfusion of 10% neutral buffered formalin for eight minutes. The brains were removed and placed into vials containing 10% neutral buffered formalin to maximize tissue fixture. The following day, brains were placed in 30% sucrose in 10% buffered formalin. Coronal sections (40 μm thick) were taken through the hippocampus using a microtome. Sections were mounted, and then counterstained with Neutral Red (1%). Slides were examined under a microscope to confirm cannula tip placement in the DH.

Data Analysis and Statistics

Data analysis and statistical tests were performed using the same programs as previous experiments. All surgeries but one successfully implanted the cannula with the injector tip located in the DH and the tip placements for animals included into the analysis are shown in Figure 12. Two rats from the un-operated preexposed-other group and one rat from the saline preexposed-other group were excluded due to procedural error. The remaining rats were distributed across the six groups as follows: 3 male and 6 female rats in the MK-801 preexposed-same group, 4 male and 4 female rats in the MK-801 preexposed-other group, 5 male and 3 female rats in the saline preexposed-same group, 3 male and 3 female rats in the saline preexposed-other group, 7 male and 4 female rats in the un-operated preexposed-same group, 2 male rats and 1 female rat in the un-operated preexposed-other group. Planned comparisons between the mean freezing levels for the saline and un-operated preexposed-same animals demonstrated the groups did not differ. Therefore, the data were collapsed resulting in the control preexposed-same group. The other planned comparison between average freezing levels for the saline and un-operated preexposed-other

animals found that these groups did not differ as well. Thus, the data were collapsed to form the control preexposed-other group. Consequently, the control preexposed-same group had 8 male and 7 female rats, while the control preexposed-other group had 5 male and 4 female rats. A 2 x 2 x 2 factorial ANOVA was run (with sex, preexposure condition and drug treatment as independent factors). Newman-Keuls post-hoc analysis was used to further examine trends found in the ANOVA.

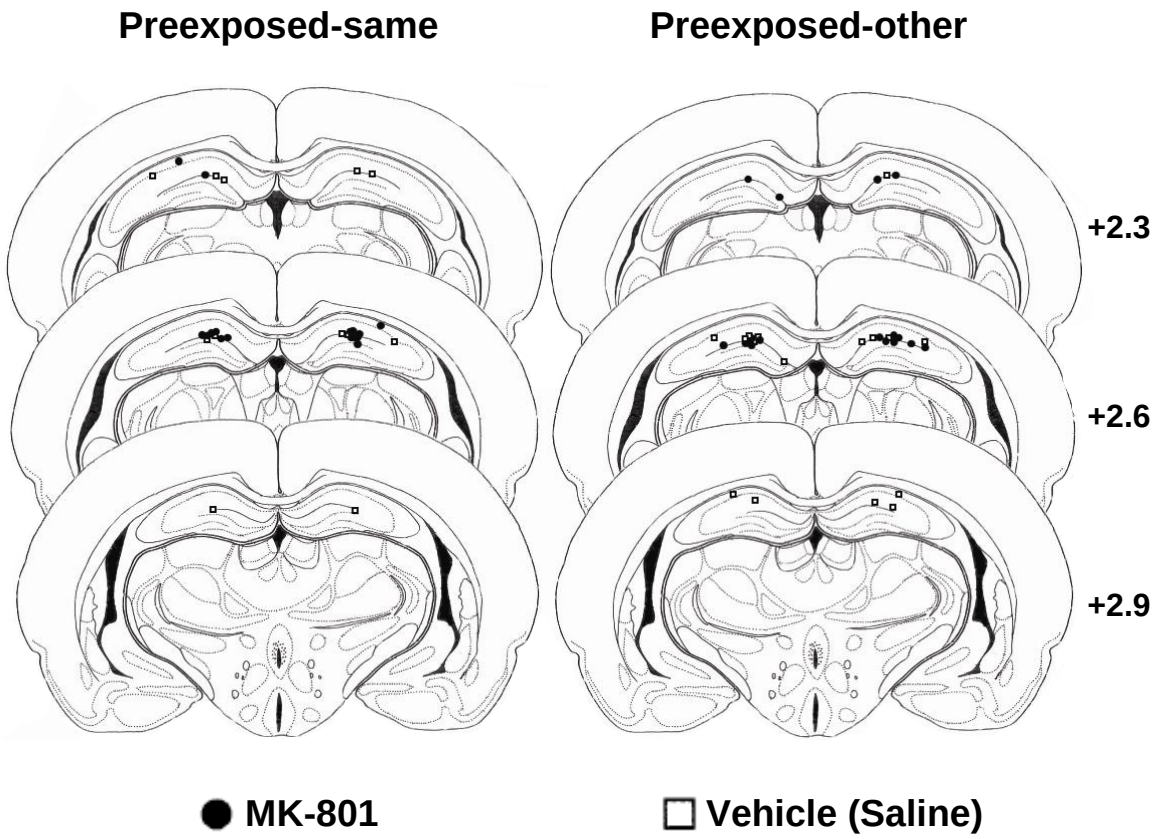

Figure 12. Schematic Representation of Injection Cannula Tip Placements in Dorsal Hippocampus for All Rats Included in Experiment 4

Left panel shows placements for preexposed-same animals, while the right panel shows placements for preexposed-other animals. The values to the right indicate the anterior position (in millimeters) of each section relative to interaural midline. Coronal brain images are adapted from the developing brain atlas of Sherwood & Timiras (1970).

Results & Discussion

The results can be seen in Figure 13, and they demonstrate the critical role NMDA-type glutamate receptors in the dorsal hippocampus play during the preexposure phase at the approximate age of emergence of the CPFE-ISD. The control group exhibits the classic CPFE-ISD, with the preexposed-same animals freezing at significantly higher levels than the preexposed-other group. In contrast, MK-801 infusion into the DH before context preexposure eliminated the CPFE-ISD.

There were no main effects or interactions involving gender (*ps*>.57). Significant main effects of preexposure condition (F (1,33) = 8.80, p<.01) and drug treatment (F (1,33) = 6.09, p<.01) were observed. More importantly, the significant preexposure condition x drug treatment interaction (F (1,33) = 4.71, p<.05) demonstrating the differential effect of context preexposure on the different drug treatment groups. Newman-Keuls Post-hoc analysis of the significant interaction revealed the control preexposed-same group (23.49 ± 4.44%) differed from all the other groups (*ps*<.01), while none of the other groups differed from each other (*ps*>.79).

The pattern of results confirms the disruption of the CPFE-ISD resulting from MK-801 infusion. Administration of the drug into the DH seems to block the facilitating effect of context preexposure as evidenced by the significant CPFE-ISD observed for the control preexposed-same group. In fact, MK-801 infusion causes the preexposed-same animals to demonstrate conditioning levels that are identical to those observed for animals that did not receive context preexposure, specifically they exhibit the immediate shock deficit.

Burman et al. (*under revision*) suggested a general role of NMDA receptors in the formation of the contextual representation at this age. The observation

that MK-801 infusion into the DH effectively blocked the facilitating effect of context preexposure on the ISD, suggests the neurons of the dorsal hippocampus play an integral role in the formation of the conjunctive representation during the preexposure phase and this mnemonic function is NMDA-dependent on PND 24, just as it is in adult rats (Stote & Fansleow, 2004). This account is consistent with the prevailing neuromolecular theory that describes the conjugation of elemental features into a unitary representation is mediated by the neural plasticity resulting from NMDA-dependent long term potentiation in the hippocampal neurons (Maren, 2001; Fanselow & Poulos, 2005). So although Pugh & Rudy (1996) reported support for the enhanced *saliency view* of the CPFE for PND 23 conditioned animals, which suggested contextual fear conditioning was mediated by the SAS, the results from this experiment clearly demonstrate the mnemonic function of the CAS is intact, and that function seems to depend upon the action of NMDA-receptors in the dorsal hippocampus. NMDA-antagonism of the DH during a more conventional paradigm would address the question of whether the SAS-inhibitory function of the CAS has developed by PND24. As only the mnemonic function of the CAS would be impaired, low levels of conditioning would suggest the inhibitory efferent projections to the SAS were functioning properly. On the other hand, if the manipulation generated significant levels of conditioning, the dissociable development of the CAS functions would be supported, where the mnemonic function would develop earlier in ontogeny than the inhibitory function. The latter account would explain the support for the *enhanced saliency view* of the CPFE at the age of emergence that was expressed in Pugh & Rudy (1996), as both the CAS and SAS could be concurrently operating.

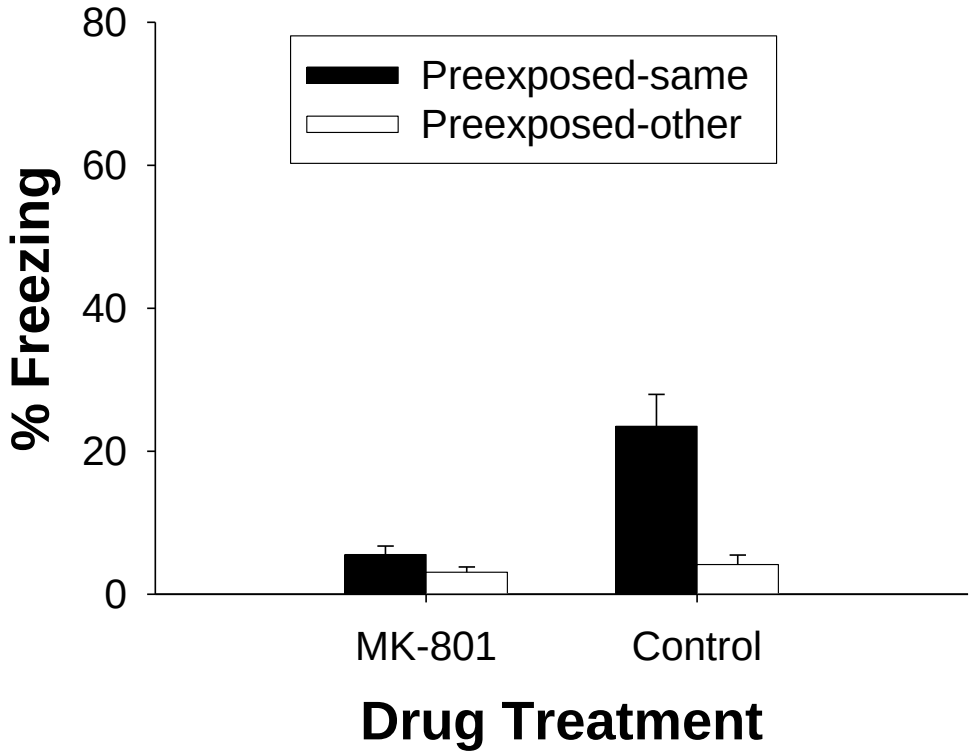

Figure 13. Effect of Drug Treatment on Conditioning Levels Generated from the CPFE-ISD Paradigm with Two Shock Training

Mean percent freezing depicted for preexposed-same (black) and preexposed-other (white) groups across drug treatment groups (MK-801 vs. Control (collapsed saline and un-operated data)). Bars represent standard errors of the mean. Either the drug MK-801 or saline vehicle was infused into the dorsal hippocampus prior to the preexposure phase. Un-operated rats were handled in a yoked manner during infusion procedure. Significant CPFE-ISD only observed for control group. The CPFE-ISD was eliminated by MK-801 infusion into the DH before the preexposure phase, suggesting MK-801 blocked the necessary formation of the conjunctive representation that is required for conditioning in the CPFE-ISD paradigm.

Chapter 6

GENERAL DISCUSSION

Summary of Findings

These experiments investigated the ontogeny of the context preexposure facilitation effect (CPFE) across the period of development when conventional contextual fear conditioning emerges in the rodent. Potential neural substrates of the CPFE at its approximate age of emergence were also examined. Experiment 1 established a preliminary developmental profile of the CPFE-ISD as the contextual fear conditioning ability emerges. PND 17 preexposed rats demonstrated low levels of conditioning regardless of preexposure condition. Relatively low levels of freezing were observed for PND 24 preexposed animals as well, and the CPFE-ISD was not observed. The CPFE-ISD had clearly surfaced by PND 31. Training parameters were manipulated in Experiments 2 & 3 to address the observed deficit in conditioning for PND 24 preexposed-same animals in Experiment 1. Consistent with the variable development account, the objective was to drive the potentially underdeveloped animals into the parametric space where a greater portion of preexposed-same animals could successfully condition to fear the context under parameters that still favored utilization of the CAS, as evidenced by low levels of conditioning for the preexposed-other group. Experiment 2 trained rats using various placement-to-shock intervals (PSIs). Varying the PSI across the developmental profile also served to examine the ontogeny of conventional (i.e., no preexposure phase and long PSIs on the training

day) contextual fear conditioning. Experiment 2A delivered the shock to rats preexposed on PND 24 after either an immediate, 10s or 30s PSI. Significant CPFEs were observed for both the immediate and 10s PSI groups. Experiment 2B delivered the shock to rats preexposed on PND 31 after either an immediate, 30s or 120s PSI, or to PND 17 preexposed rats after either an immediate or 120s PSI. A significant CPFE was only observed for PND 31 preexposed animals that were given an immediate shock. PND 17 preexposed animals did not exhibit fear conditioning across all preexposure and PSI groups, while high levels of freezing are reported for both PND 24 and 31 preexposed animals following the longer PSI intervals (30s & 120s), regardless of preexposure condition. These results indicate the conventional contextual fear conditioning ability rapidly emerges between PND 17 and PND 24. As Experiment 2A demonstrated a significant CPFE-ISD for PND 24 preexposed animals, additional training parameters were manipulated to further characterize the nature of the CPFE-ISD across the ontogenetic profile. Specifically, Experiment 3 increased the number of immediately delivered shocks to two instead of one. PND 17 preexposed animals continued to freeze at low levels. A similar CPFE-ISD was observed for PND 31 preexposed animals, while multiple shocks may have enhanced the CPFE-ISD at PND 24 to match that of the older animals. As the potential difference in the neural systems used to mediate the CPFE between young developing rats and mature adults needed to be addressed empirically, Experiment 4 investigated the role of NMDA-type glutamate receptors in the dorsal hippocampus during the preexposure phase of the CPFE-ISD paradigm at the approximate age of emergence (PND 24). Infusion of the NMDA-receptor antagonist MK-801 abolished the facilitating effect of context preexposure. Thereby, successful contextual conditioning in the CPFE-ISD paradigm

at the age of emergence depends upon the activation of NMDA-receptors in the dorsal hippocampus which mediates the formation of the conjunctive representation during the preexposure phase. The pattern of results across the study reinforce the ascription of PND 24 as the approximate age of emergence for contextual fear conditioning, and demonstrate the hippocampal-dependent CAS mnemonic function can support learning under the proper training conditions at that age.

As no significant freezing levels were ever observed for animals infused with MK-801 in Experiment 4, a performance account may explain the deficits in conditioning that could be falsely attributed to an impaired mnemonic function. The performance account argues the observed low levels of conditioning could be confounded by lasting drug effects or potential brain damage caused by the drug action, which may have impaired the rats' ability to freeze during the test phase. As a result, one may mistakenly conclude the essential NMDA-dependent mnemonic role of the neurons in the dorsal hippocampus in the formation of the conjunctive representation. As the neural substrates for contextual fear conditioning at the age of emergence have never been examined, this potential complication may lead to the erroneous conclusion of similar biological mechanisms underlying contextual fear conditioning between mature adults and developing rats. A recently completed experiment that was not included in this thesis (but will be published with these experiments) confirms the observed deficit was in the learning that takes place during preexposure and not due to impaired performance across the phases of the paradigm. Bilateral infusions of MK-801 into the DH two hours after the preexposure phase control for the potential lasting or damaging effects of the drug, while allowing for the formation of the conjunctive representation and potential NMDA-dependent

consolidation that may occur immediately after its construction. The preexposed-same animals froze significantly more than the preexposed-other group, so that the CPFE-ISD was observed even though the preexposed-same animals received infusions of MK-801 after preexposure. This performance control is critical, and with it, the proposed impairment of the NMDA-dependent mnemonic function of the DH following MK-801 infusion is validated.

Ontogenetic Comparisons

Successful contextual fear conditioning across PND 24-26 and PND 31-33, along with the deficits observed for the animals trained on PND 18, is consistent with previous research identifying PND 23 as approximate age when this type of fear conditioning emerges in rodents (Burman et al., *under revision*; Rudy, 1993; Rudy & Morledge, 1994; Stanton, 2000). A similar ontogenetic profile of conventional (no preexposure phase) contextual fear conditioning following a long PSI and 24 hour retention interval in Rudy & Morledge (1994) is observed when comparing the preexposed-other groups across the long PSIs in Experiment 2. Nonetheless, it must be noted that only the animals trained on PND 18 and 32 received comparable PSIs of 120s, while the longest PSI for PND 25 trained animals was 30s in this study. However, Burman et al. (*under revision*) reported similar levels of conditioning for PND 24 trained preexposed-other animals across 30s and 120s PSIs. Therefore, it seems safe to conclude that the results of this study verify conventional contextual fear conditioning emerges rapidly between PND 17 and PND 24.

The observed CPFE for animals preexposed on PND 24 and PND 31 is consistent with the findings that demonstrate a general facilitation of contextual fear conditioning after context preexposure at these ages (Burman et al., *under revision*;

Rudy & Morledge, 1994; Rudy & O'Reilly, 1999). A significant CPFE-ISD around PND 24 replicates results from Burman et al. (*under revision*). However, Rudy & Morledge (1994) and Rudy & O'Reilly (1999) observed the CPFE (for PND 23 & 30 preexposed animals, respectively) using subtle yet significant differences in protocol, namely a 120s PSI and a 10 min retention interval (the time between conditioning and testing). The relevance of these slight distinctions becomes apparent when the data from Pugh & Rudy (1996) are considered.

Pugh & Rudy (1996) performed a series of experiments preexposing PND 18 and 23 aged animals to a variety of conditions, which were intended to further examine trends observed in Rudy & Morledge (1994). The first interesting observation did not involve preexposure of any sort, but characteristics of conditioning context. Using a 120s PSI standard contextual fear conditioning protocol, substantial levels of conditioning were observed 24 h later for PND 18 animals trained in a black "salient" context, while the clear context group did not exhibit robust conditioning. A subsequent investigation preexposed PND 18 and 23 animals to both black and clear conditioning chambers as well as their individual elements (see Figure 5 in General Introduction). Similarly increased levels of freezing were observed within both age groups for animals preexposed to either the entire black context or the separated features of the black context when compared to the handled controls. Meanwhile, reintroduction to the clear training context produced similarly high levels of freezing for PND 23 animals preexposed to the context or its features, while PND 18 preexposed animals did not express much fear regardless of their preexposure condition. These results supported the *enhanced saliency* of individual features explanation for the CPFE. The next experiment in Pugh & Rudy (1996) conditioned

PND 18 and 23 aged animals to either a black or clear context and the animals were tested 24 h later in either the same or opposite context. Testing in the black context produced high levels of freezing for both age groups, even for those conditioned in the clear context. Meanwhile, testing in the clear context did not elicit robust fear responses for either age or training context condition. Taken together, it seems highly probable that the conditioning exhibited by the PND 18 animals was supported solely by the SAS, as CAS-dependent conditioning could not be observed for those animals when the CPFE-ISD paradigm was used throughout this study. The remarkable differences in conditioning levels between the black and clear contexts observed at this age supports the enhanced saliency of the black context and suggests some feature of the black context was successfully associated with the shock or was capable of inducing fear without any association at all, as evidenced by the increased freezing levels for the PND 18 trained animals that were conditioned in the clear context but tested in the black context relative to the PND 18 trained animals that were conditioned and tested in the clear context, a group that demonstrated almost no fear. The same enhancement of freezing levels following the testing switch to the black context was observed for PND 23 trained animals. As the *enhanced saliency view* of the CPFE was maintained at the generally accepted age of emergence (PND 23) for contextual fear conditioning, the data from Pugh & Rudy (1996) suggest it is either supported by the SAS or that the SAS-inhibiting function of the CAS may not have developed to functional levels. As the mnemonic function of the CAS has been clearly demonstrated by PND 24 in Burman et al. (*under revision*) and throughout this study, with substantial evidence reported in Experiment 4, the conventional conditioning reported at that age is most likely supported to a large degree by the CAS.

The potentially latent emergence of the SAS-inhibitory function complicates the determination of the relative contribution of the SAS and CAS in conventional context fear conditioning paradigms. If the development of functions was truly dissociated, it would be impossible to attribute this contextual fear conditioning for these younger rats to the CAS-mediated formation of the conjunctive representation, as is the case in adults. Therefore, the general conditioning and the observed CPFE around PND 24 in Rudy & Morledge (1994) may have been supported by both associative systems, whereas the CPFE-ISD reported in this study clearly relies on the CAS. Rudy & O'Reilly (1999) reported evidence that supported the *conjunctive view* of the CPFE for rats preexposed on PND 30 (see Figure 4 in General Introduction), so SAS-inhibition seems to have developed to functional levels by that age. Therefore, the general CPFE reported in Rudy & O'Reilly (1999) and the CPFE-ISD for PND 31 preexposed animals in this study probably share underlying mechanistic processes (both functions of the CAS). The approximate age of emergence (PND 24) is the most intriguing when considering the potential effects of variable emergence of CAS and SAS function and the resulting extent to which the different associative strategies (and respective underlying neural substrates) are utilized in contextual fear conditioning. NMDA-antagonism of the hippocampus around this age in conjunction with a more conventional conditioning paradigm would address whether the SAS-inhibitory function has developed.

Adult Comparisons

Interesting insights can be garnered when comparing the results of this study with those from adult studies involving the CPFE-ISD. When adult rats were given a 2 minute preexposure to the shock context a day prior to conditioning, the

CPFE was not observed if the shock was delivered immediately upon placement, but was observed if a PSI of 9s was used (Fanselow, 1986). Subsequent experiments demonstrated a lack of CPFE for immediate, 81s, and 162s PSIs, while significant CPFEs were observed after 9s and 27s PSIs (Fanselow, 1990). It was argued that a certain amount of time (8s) preceding the shock was needed for the context memory to be retrieved and available for association. However, the CPFE could be demonstrated following an immediate shock if multiple preexposures were given (Rudy & O'Reilly, 2001). A single 4 min preexposure, followed by a number of 40s exposures seemed to establish an association between the transport cues (characteristics of the black ice bucket used to transport the subject from their home cage) and the preexposure context. In fact, a subsequent manipulation dissociating the preexposed context from the conditioning context (the two were different) demonstrated this paradigm caused the rat to associate the shock with the retrieved memory of the preexposed context and not to any facet of the actual conditioning context. Therefore, the paradigm established a reliable association between the transport cues and the preexposed context, and during the training phase, those transport cues retrieved the preexposed context memory which could then be associated with the immediate shock (Rudy & O'Reilly, 2001). Interestingly, this study demonstrated a significant CPFE-ISD for animals preexposed on PND 24 and 31 following a *single* transport and preexposure. Either the younger aged rats can retrieve the context memory following insertion into the chamber more rapidly than adults, or the CPFE-ISD paradigm used in the study was successful in establishing the transport-preexposed context association. Apparently, the paradigm (single 5 min preexposure with transport to and from within a small lexan chamber surrounded by orange paper) used in this study successfully

established an association between the preexposed context and the transport cues; an association which caused the retrieval of the context memory prior to conditioning. The exact nature of these associations cannot be determined, as the preexposed-other animals (which were transported in identical containers) were not tested in the context they were preexposed in. If the conditioning context is indeed irrelevant when using an immediate shock, then the preeexposed-other animals would be expected to demonstrate fear upon reintroduction to their preexposed context. Conversely, there is a chance that the transport cue-preexposed context association established in this study after a single preexposure requires the training context to match the preexposed context for successful conditioning to occur. The transport cue-preexposed context association may be weak following a single preexposure, but the split second re-exposure to the identical context before immediate shock delivery completes the retrieval process. In this case, deficits in the preexposed-other group would be expected if they were tested in their preexposed context. Either way, this paradigm seems to successfully establish the transport cue-preexposed context association after a single preexposure that is required for a significant CPFE-ISD to be observed.

Pharmacological investigations suggest the mnemonic function of the CAS depend upon the proper functioning of NMDA-receptors in the dorsal hippocampus throughout the rat's life. Intracerebroventricular (ICV) infusion of the NMDA antagonist APV before preexposure blocked its facilitating effect on contextual fear conditioning in adults (Stote & Fanselow, 2004). Although ICV application of the drug may have had widespread effects, the main action of the drug is argued to be confined to proximal NMDA receptors in hippocampal tissue (Stote & Fanselow, 2004). The present study extends the essential mnemonic functional role of

NMDA-receptors in the dorsal hippocampus during the preexposure phase to animals during the weanling period of development. Furthermore, widespread effects are less likely as the NMDA antagonist MK-801 was infused directly into the DH. So although the CAS may not always be used in contextual fear conditioning, throughout the life of the animal its utilization seems to depend on the function of NMDA-receptors in the DH that mediate the formation of the conjunctive contextual representation.

Theories of Hippocampal Function

Morris (2007) outlines and discusses a number of theories on hippocampal function. Squire's *declarative memory theory* emphasizes that the primary function of the hippocampus is in memory. The hippocampus is a part of the medial temporal lobe memory system that has a time-limited role in the formation and initial storage of declarative memories, or the memory of facts and events that, in humans, can be consciously recalled. In the present study, the context memory, and subsequent context-shock association would be the declarative memory that over time would be consolidated into neocortex. O'Keefe and Nadel's *cognitive map theory* supposes the hippocampus to be the operating "locale" system and storage site of spatial maps. Having evolved to assist spatial navigation, the "locale" system organizes the representation of perceived stimuli with respect to a spatial framework, or cognitive map. The "locale" system operates rapidly and automatically during exploration. A hippocampus-independent "taxon" system can also be used for spatial learning, but is much slower and goal-directed. Successful contextual fear conditioning then, encodes the unified context representation as a spatial map that relates the arrangement of particular features. Sutherland & Rudy's configural-association theory suggests the hippocampus functions to build representations of combinations of elements as unitary

"configural" associations and assists the eventual storage of these unitary representations in neocortex. O'Reilly and Rudy revised the theory by describing the formation of *conjunctive representations*. In this interpretation (and similar to the *cognitive map theory*), the hippocampus is involved in the type of conjunctive learning that occurs rapidly and automatically as a consequence of exploration, while another slower, more deliberate form of conjunctive learning (not to be confused with SAS) induced by problem-solving demands is hippocampus-independent. Since it is "spontaneous" rather than directed at particular problem (reinforcement contingency), the CPFE-ISD relies upon the hippocampus-dependent conjunctive learning mechanism which automatically combines the features of the context into a unitary conjunctive representation. Taken together, these theories of the hippocampus describe its integral role in the acquisition of the context memory during the preexposure phase in the CPFE-ISD paradigm.

Ontogeny of the Hippocampus and Associated Memory Systems

Potential differences exist between the strategies and subsequent neural substrates adult and developing rats use in this CPFE variant of contextual fear conditioning. Stanton (2000) details three basic and interacting memory systems: (1) the sensorimotor system: which is involved with particular sensory-effector pathways as well as a basic organization of behavior, (2) the affective system: which concerns hedonic or motivational states, and (3) the cognitive system: which encodes elaborate mental representations of features and relations. The systems can function independently, but may also interact to influence associative learning within a given system as well as produce a specific behavioral response. Furthermore, these systems and their interactions undergo variable development throughout ontogeny, with the

sensorimotor and affective systems emerging before the cognitive system. This developmental dissociation can be observed when comparing auditory cue fear conditioning (observed at PND 18) with contextual fear conditioning (observed at PND 23; Rudy, 1993). In theory, auditory cue fear conditioning only requires the sensorimotor system and the amygdala-dependent affective system, while contextual fear conditioning requires both aforementioned systems along with the hippocampus-dependent (CAS) cognitive system (Stanton, 2000). The late development of the hippocampus (which undergoes substantial development from birth to PND 30) or the underdeveloped interactions between the cognitive system and the other systems may explain the developmental delay for the emergence of the contextual fear conditioning ability (Stanton, 2000; Rudy, 1993; Rudy & Morledge, 1994). Analogously, the CAS may be underdeveloped while the SAS is functioning properly (Rudy, 1993). This concurrent yet variable development of systems and their interactions may impact the nature of the contextual fear conditioning ability as it emerges. Specifically, the CAS clearly develops its mnemonic function (evidenced by successful conditioning in the CPFE-ISD paradigm) by PND 24, but the level of development of the interactions that mediate the inhibition of the SAS functioning is unknown. Therefore, as the ability emerges, the rat may be utilizing both associative strategies in conventional contextual fear conditioning tasks, and the conditioned response may be an artifact of integrative control.

Conclusion

The ontogeny of the context preexposure facilitation effect that attenuates the immediate shock deficit (CPFE-ISD) parallels the development of the conventional contextual fear conditioning ability by rapidly emerging between PND 17 and PND

24, suggesting similar neural mechanisms may underlie both phenomena. Proper mechanistic investigations must account for the two distinct and competing associative strategies (SAS vs. CAS) and related neural substrates that can be used to support conditioning. Using the CPFE-ISD paradigm, which requires CAS utilization for successful conditioning, this study provided evidence that the mnemonic function of the CAS emerges around PND 24. Furthermore, the formation of the conjunctive representation seems to be rely on the activity of NMDA-type glutamate receptors in the dorsal hippocampus at this age. Therefore, hippocampal LTP seems to be the neuromolecular mechanism behind the mnemonic function of the CAS both early in ontogeny and in the mature adult (Young et al., 1994; Stote & Fanselow, 2004). The results of Pugh & Rudy (1996) suggest the SAS may contribute to conventional contextual fear conditioning early in ontogeny, and that the SAS-inhibitory function of the CAS may emerge later than the mnemonic function. Antagonism of the NMDA-receptors in the dorsal hippocampus for PND 24 rats conditioned with a more conventional paradigm would assess the possible dissociated emergence of CAS function.

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