The Ecology and Ontogeny of Odor Fear Learning

by

Patricia A. Kabitzke

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Michael Lewis, Ph. D

Date — Chair of Examining Committee

Maureen O'Connor, Ph. D.

Date — Executive Officer

Gordon Barr, Ph. D.

Christopher Braun, Ph. D.

Donald Wilson, Ph. D.

Frances Champagne, Ph. D.

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

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Advisor: Dr. Christoph Wiedenmayer

Predator odors have been found to induce unconditioned fear in adult animals and provide the opportunity to study the mechanisms underlying unlearned and learned fear. The clinical application of this research is to explore the causal relationships between aversive events and psychopathologies such as PTSD. However, trauma often occurs early in life but most current investigations use adult animals in paradigms that employ stimuli with little ecological relevance in limited environmental contexts. Additionally, predator threats change across an animal's lifetime, as do abilities that enable the animal to learn or engage in different defensive behaviors. Thus, the first objective of this study was to determine the combination of factors that successfully induce unlearned fear to predator odor across development. Cat odor effectively induced fear-related behavior across development using the behavioral measure of freezing, especially in infant (PN14) and juvenile (PN26) rats. Once these parameters were understood, they were exploited to develop a learning paradigm to predator odors that could be used in early life. Cat odor produced unlearned, innate fear in infant and juvenile rats, but contextual fear learning occurred only in juveniles. The mechanisms underlying the development of this learning in early life were then explored. It was hypothesized that contextual fear learning is

mediated by norepinephrine. Systemic injections of the β-adrenergic antagonist propranolol before exposure to the cat odor reduced the unlearned fear response and memory acquisition whereas injection of propranolol after exposure to cat odor inhibited contextual fear learning in juvenile rats. We suggest that NE mediates the formation of contextual fear memories by activation of the transcription factor CREB in the hippocampus in juveniles but not in infants. Levels of phosphorylated CREB (pCREB) were increased in the dorsal and ventral hippocampus in juvenile, but not infant, rats that had been exposed to cat odor but not in animals exposed to a control odor. Further, propranolol blocked these increases in pCREB. Taken together, these results indicate that, although innate fear occurs within the neonatal period, contextual fear learning is a relatively late-occurring event, is hippocampal dependent, and mediated by norepinephrine.

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ABBREVIATIONS

BLA	Basolateral amygdala
CA1	Cornu Ammonis area 1
cAMP	Cyclic adenosine monophosphate
CPFE	Context preexposure facilitation effect
CRE	cAMP response elements
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DH	Dorsal hippocampus
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
i.p.	Intraperitoneal
LC	Locus coeruleus
LTM	Long-term memory
LTP	Long-term potentiation
MeA	Medial amygdala
NE	Norepinephrine
pCREB	Phosphorylated cAMP response element binding protein
PKA	Protein kinase A
PN	Postnatal
PTSD	Post-traumatic stress disorder
STM	Short-term memory
TMT	Trimethylthiazoline
US	Unconditioned stimulus
VH	Ventral hippocampus

Chapter 1: Introduction

Fear in humans is often defined as the response to a present, definable threat while anxiety is the anticipation of an amorphous, perhaps imagined, danger. The concept of fear has been theoretically extended to animals in order to describe a suite of behavioral responses to aversive or threatening stimuli in order to model generalized anxiety in humans (Davis, 1992) (Table 1). Although the terms "fear" and "anxiety" are often used interchangeably in the animal literature, tests of anxiety most often place the animal on an elevated plus-maze or in an open field (Pellow et al., 1985; Hale et al., 2006) whereas tests of fear utilize a negative or threatening stimulus (Blanchard et al., 1986b).

Table 1
Behavioral measures of fear in animals compared to measures of human generalized anxiety.
From: Davis: ANNU REV NEUROSCI, VOL 15 (1992) 353-375.

Measures of fear in animal models	DSM criteria - generalized anxiety
Increased heart rate	Heart pounding
Decreased salivation	Dry mouth
Stomach ulcers	Upset stomach
Respiration change	Increased respiration
Scanning and vigilance	Scanning and vigilance
Increased startle	Jumpiness, easy startle
Urination	Frequent urination
Defecation	Diarrhea
Grooming	Fidgeting
Freezing	Apprehensive expectation

The negative stimulus that has most often been studied in both humans and animal models is electric shock, which produces pain. From a methodological standpoint, shock is appealing because the experimenter can accurately control the duration and intensity of the stimulus. But, aside from pain, what does this stimulus signify to the animal? In humans, shock elicits a physiologically similar reaction across individuals and increases defensive reactions regardless of accompanying, even pleasant, cues (Bradley et al., 2005). The meaning of a shock to an animal is even less clear.

Recently, more attention has been given to other fear-inducing stimuli. Specifically, predator and predator odor cues are used to produce both learned and unlearned fear in laboratory animals (Do Monte et al., 2008; Takahashi et al., 2008; Mackenzie et al., 2010). Though natural predator odors are extremely difficult to quantify and control experimentally, predator odors represent an obvious threat to the animal. Such stimuli result in robust, observable behaviors (i.e. freezing) with roots in evolution and a basis in life history and offer the unique opportunity to integrate the studies of ecology and neuroscience. Using this integrative approach, a more complete understanding of biological systems is possible as data is gathered not only to answer how, but also why a behavior is produced (Kavaliers and Choleris, 2001). The field is changing in terms of how fear is studied and treated as predator-prey interactions are used to study how aversive events may affect long-term neurobehavioral processing (Foa et al., 1992; Koolhaas et al., 1997; Sanchez et al., 2001). One intriguing area of exploration is the use predator odor cues to induce unlearned and learned fear across development. Through this research, predator odors provide the unique opportunity to study the mechanisms underlying these abilities.

I. Predator Odor Cues: Ecology

Cat odor cues are used more often than a live cat in studies to elicit fearful or defensive behaviors in rats. These cat odor cues include cat collars, cloths rubbed on cats, cat fur, cat bedding, cat urine, cat feces, and soiled cat litter (Dielenberg and McGregor, 2001; Blanchard et al., 2003b; Li et al., 2004). "Fear" and "defensive" are often used interchangeably in the literature to describe the response of rats to cat odor cues, a distinction can be made on the basis of what behavior is elicited. Complete immobility, or freezing, is frequently termed fear behavior while defensive behaviors include avoidance, locomotion reduction, and risk-assessment behaviors (Blanchard and Blanchard, 1971; McGregor et al., 2002; Hubbard et al., 2004). Odors provide particularly salient cues to rodents because olfaction is a relevant, primary sense modality used for communication, orientation, and risk-assessment (Brown, 1985; Kats and Dill, 1998; Lavenex and Schenk, 1998). The olfactory system is one of the oldest mammalian sensory systems, phylogenetically, and develops early in ontogeny.

The variety of unlearned fear-related responses that rats display to different predator odorants are contingent on several factors, some known and some unknown. For example, it is known that stimuli of different kinds and intensity can elicit behaviors of different type or intensity that are specific to the stimulus (Dunn, 1987; Mormede et al., 1990; Takahashi et al., 2005). The interactions between a variety of ecologically-relevant fear-inducing odor stimuli and their potency on unlearned fear-related responses, however, are not known. In fact, there are conflicting reports of innate prey responses to predator odors. Differences between individual investigator's interpretation of behavior are always a factor (Burwash et al., 1998), however this may additionally be caused by

the possibility that a range of responses exist in the exposed population (Cohen et al., 2006).

Many studies have used trimethylthiazoline (TMT) as the stimulus "predator odor." TMT is a commercially-available synthetic compound which includes extracts from fox feces and, at times, elicits fear-related behaviors such as avoidance and freezing. However, TMT likely does not communicate the same threat as cat odors, possibly because of its synthetic nature or because scatological cues are less predictive of actual threat (Staples et al., 2008). Cats are common predators of rats and these two genera co-evolved so that rats respond to cat odors in adaptive ways. Indeed, avoidance to cat odor has been observed universally across various rat strains, indicating its relevance as fear-inducing stimulus (Dielenberg and McGregor, 2001). This "evolutionary adaptive roots" hypothesis is supported by data showing that only cat odor, and not TMT, elicits defensive behaviors such as risk-assessment (i.e. head-up, flatback approach, stretch attend) and activates necessary sites within the hypothalamus and brainstem (Dielenberg and McGregor, 2001; Staples et al., 2008) (Table 2).

Although research with adult animals has explored various stimuli, limited research has investigated how environmental factors may influence behavioral responses to predator stimuli. Differences in the unlearned fear response may also occur when the environment changes, not only in terms of size, but in features. For example, gerbils prefer to approach the predator to gain access to a shelter (Ellard, 1993). In response to cat odor, it appears that the rat's natural pattern is to terminate exploratory and grooming behaviors, retreat to a location of lower danger (i.e., a hide box) and then engage in freezing and 'head out' behavior in order to decrease conspicuousness and supervise the

Table 2

Exposure to different predator odors changes defensive repertoire.
From: Blanchard, Griebel, and Blanchard: PROGRESS IN NEURO-PSYCHOPHARMACOLOGY AND BIOLOGICAL PSYCHIATRY, VOL 27 (2003a) 1177-1185.

Rat defensive behavior	Live cat	Cat fur/skin odor	TMT
Avoidance	Yes (Blanchard & Blanchard, 1971, 1989; Blanchard et al., 1976, 1989a,b)	Yes (Blanchard et al., 1990, 1991, 1993, 2001b; Dielenberg & McGregor, 1999; McGregor et al., 2002)	Yes (Burwash et al., 1998a; Vernet et al., 1992); No (Burwash et al., 1998b, field study)
Eating reduction	Yes (Blanchard & Blanchard, 1989; Blanchard et al., 1989a,b)	Yes (Blanchard et al., 1990, 1991, 1993)	Yes (Burwash et al., 1998a)
Freezing/immobility	Yes (Blanchard & Blanchard, 1971, 1989; Blanchard et al., 1976, 1989a,b)	Yes (Blanchard et al., 1990, 1991, 1993, 2001; Dielenberg & McGregor, 1999; McGregor et al., 2002)	Yes (Hotsenpiller & Williams, 1997; Wallace & Rosen, 2000); No (Morrow et al., 2000a)
Locomotion reduction	Yes (Blachard & Blanchard, 1971, 1989; Blanchard et al., 1976, 1989a,b)	Yes (Blanchard et al., 1990, 1991, 1993, 2001b; Dielenberg & McGregor, 1999; McGregor et al., 2002)	No (Morrow et al., 2000a)
Nondefensive behavior reduced	Yes (Blachard & Blanchard, 1989; Blanchard et al., 1989a,b)	Yes (Blanchard et al., 1990, 1991, 1993, 2001b)	No (Morrow et al., 2000a)
Risk assessment	Yes (Blachard & Blanchard, 1989; Blanchard et al., 1989a,b)	Yes (Blanchard et al., 1990, 1991, 1993, 2001b; Dielenberg & McGregor, 1999; McGregor et al., 2002)	No (McGregor et al., 2002)
Anxiogenic in elevated plus maze	Yes (Adamec & Shallow, 1993; Adamec et al., 1997, 1998, 1999a,b)	Yes (Dielenberg & McGregor, 1999; McGregor et al., 2002; Zangrossi & File, 1992a,b)	No (McGregor et al., 2002)
Supports conditioning	Yes (Blanchard & Blanchard, 1989; Blanchard et al., 1989a,b) (indirectly assessed by long-term behavior change in cat-exposure context)	Yes (Blanchard et al., 2001b; Dielenberg & McGregor, 1999; McGregor et al., 2002)	No (Blanchard et al., 2001b; McGregor et al., 2002; Morrow et al., 2000a; Wallace & Rosen, 2000)

threat, respectively (Dielenberg and McGregor, 2001). This repertoire is interrupted only with occasional rearing and flatback-approaches (Dielenberg and McGregor, 2001). Both freezing and avoidance responses are available so that the expression of one behavior does not inhibit the other (de Oca et al., 2007). Across different prey species, for example squirrels, fleeing and concealment are dominant strategies (Owings and Coss, 1977; Dill and Houtman, 1989) and strong, active defensive responses occur across ontogeny (Hanson and Coss, 1997). In rats, flight is more likely than freezing when escape is provided (Blanchard et al., 1986a). Thus, the behaviors that are expressed in response to threat are flexible to present conditions (Tolman, 1932; Blanchard et al., 1976).

The response to predator odor may also change throughout development as physical abilities increase and ecological threats change, but few studies have investigated the effect of predator odors during early ontogeny. Postnatal day 14 (PN14), rats freeze more in the presence of an adult male rat compared to scatological cat cues whereas juvenile, PN26, rats show the opposite pattern of freezing (Wiedenmayer and Barr, 2001). The ethological explanation for these differences during development is that infants are under predation risk from adult males that engage in infanticide while post-weaned juveniles are predated on by cats (Wiedenmayer and Barr, 2001). Thus, these differences in fear behavior are adaptive as animals that are able to innately respond to specific threats increase their survival and subsequent fitness.

II. Contextual Conditioning

An animal's ability to respond appropriately to a severe threat has ultimate survival advantages by facilitating escape or decreasing conspicuousness (Kavaliers and

Choleris, 2001). Whether the animal attempts to escape or freezes to decrease detection may depend on the environmental context where the predator is encountered. For example, the distance between the prey and predator and features of the environment (i.e. hiding places) contribute to the behavioral response. Altering the behavioral response according to changing conditions can be adaptive, such as when the response provides survival benefits to the individual by informing future encounters (Lima, 1998; Ohman and Mineka, 2001; Wiedenmayer, 2004). The underlying mechanisms that explain experience-dependent behavioral flexibility involve learning processes such as fear conditioning. Fear conditioning requires memory acquisition, consolidation, and retrieval of the association. Contextual odor conditioning occurs when an unconditioned stimulus (US) odor is associated with the context where it was encountered, and cued conditioning occurs when the US is associated with a neutral, conditioned stimulus (CS). Under natural conditions, contextual fear conditioning is adaptive as animals able to remember specific locations where predator odor was encountered increase their survival and subsequent fitness.

A variety of predator odor cues may elicit an unlearned fear-related response. However, the stimulus must have predictive power to promote learning. It has been suggested that the odor of cat fur/skin predicts the presence of the predator more strongly than "scatological" cues (Blanchard et al., 2003a). Because cats spend a large part of their waking time budget to oral grooming (Eckstein and Hart, 2000), encountering the odor of cat fur/skin may indicate the more immediate presence of a cat. Indeed, cat and natural fur/skin odors support contextual fear conditioning in adult rats – altering their behaviors in that context for long periods of time after a single exposure (Blanchard and Blanchard,

1989; Dielenberg and McGregor, 1999; Blanchard et al., 2001; Wiedenmayer, 2004). Urine or fecal odor informs the rat that a cat has been historically present and may be perceived as less of a threat. In one study, cat fur and feces both elicited unconditioned fear but only fur promoted contextual conditioning in adult rats (Blanchard et al., 2003a). This may be another reason why TMT loses predictive power for a rat. For example, when exposed to an unworn cat collar, a cat collar containing cat odor, TMT, or triethylamine (fishy odor), rats avoided all of these odors but only cat odor elicited unique defensive responses during exposure and only the cat odor-exposed animals exhibited conditioned contextual fear the following day (McGregor et al., 2002).

In young rats, the interpretation of an odor can vary as ecological threats change and as young animals unable to associate an odor with a context gain this ability. Very young rat pups (< PN10) are able to perform cued learning tasks but this learning is idiosyncratic as an aversive stimulus (i.e. shock) promotes approach behaviors to a neutral odor early in life but promotes avoidance behaviors as the animal develops (Sullivan et al., 2000a). Although cued conditioning to predator odors has been demonstrated in neonatal rats (Moriceau et al., 2004), contextual conditioning to shock is not supported in the young rat until it weakly emerges around PN18 (Rudy and Morledge, 1994). It is not clear at what age young animals can be contextually conditioned to predator odor. One suggestion is that early learning deficits are not due to the inability to acquire a task, but are instead the result of infantile amnesia, or the rapid forgetting by pre-weanlings unless constant reminders are given (Campbell and Spear, 1972; Rudy and Morledge, 1994). The most capable learners of simple avoidance

learning tasks appear to be adolescents (Spear and Brake, 1983). This may be adaptive as animals at this age disperse and are faced with the most challenges to their survival.

Behavioral Repertoire during the Contextual Test

Features of the environment can influence the expression of fear during re-exposure to the context where an US was encountered. The rat's typical strategy is to hide and then display freezing and vigilant risk-assessment behaviors during the contextual test (Blanchard and Blanchard, 1989). Without somewhere to hide, it's typical strategy is altered and other aspects of their behavior are altered as well (de Oca et al., 2007). A rat without a refuge in a context it has learned is dangerous will display a reduction in freezing behavior compared to a rat that is provided a shelter. Instead, behaviors like hyperactivity may take the place of avoidance, freezing, and risk-assessment.

The Context Preexposure Facilitation Effect (CPFE)

Preexposure to the context the day before training may be necessary to achieve contextual conditioning. Preexposure allows the animal to sample the environment and develop a representation of it, which can then be associated with the US (i.e., shock, predator odor). For example, Fanselow found that rats did not display fear behaviors (i.e., freezing) when returned to the context 30 minutes after shock unless they had been briefly exposed to the conditioning context the day before (Figure 1) (Fanselow, 1990). This development in the contextual conditioning paradigm represented a leap in methodology from Fanselow's earlier studies, in which adult animals were unable to

achieve contextual conditioning unless the contextual test took place at 24 hours after training (Fanselow, 1980).

Conditioned freezing in young rats is enhanced when animals are preexposed to the context (Pugh and Rudy, 1996). Though this does not prove that preexposure is necessary in all cases, it does suggest that preexposure to the context can facilitate contextual learning even in animals which eventually learn without it.

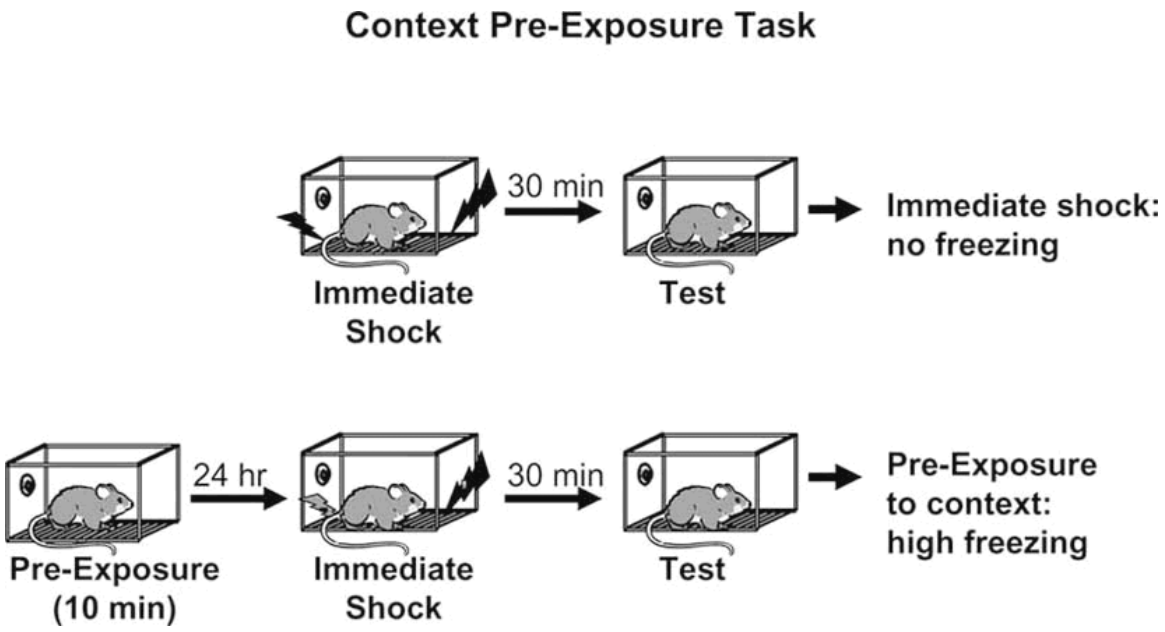


Figure 1. Context pre-exposure task. One example of a contextual fear conditioning paradigm that is hippocampal-dependent. As opposed to typical contextual conditioning, here context pre-exposure temporally disconnects the acquisition of the context from its association with shock. Rats which were given a shock immediately after introduced to the context displayed low levels of freezing. However, rats preexposed to the context 24 hours before being shocked in that context demonstrated high levels of freezing.
From: Josselyn and Nguyen: CURR DRUG TARGETS CNS NEUROL DISORD, VOL 4 (2005) 481-497.

III. Neural Substrates Underlying Fear Conditioning

The neuroanatomy of the classic shock-tone fear circuit is well understood. In simplistic terms, US sensory information from the dorsal horn of the spinal cord is relayed by the cortex and thalamus to the lateral amygdala, this information is then projected to the central amygdala, which mediates the fear response via the periaqueductal gray, activating the caudal pontine nucleus of the reticular formation and spinal motorneurons in the ventral horn of the spinal cord, producing fear-potentiated startle and freezing (Fendt and Fanselow, 1999; LeDoux, 2000). CS information (i.e. tone) is also transmitted to the lateral amygdala, and it is the convergence of US-CS information in this area during training that is responsible for the plasticity of conditioned learning (LeDoux, 2000) (Figure 2).

When the CS is the context, the hippocampus becomes critical. It projects to the basal and accessory basal nuclei of the amygdala, which subsequently projects to the central amygdala, and the central amygdala to the brainstem (LeDoux, 2000) (Figure 2). The region of the hippocampus that is involved in contextual conditioning remains under investigation. For example, pre-training lesioning of the ventral hippocampus (VH) attenuated contextual learning in response to cat odor while there was no effect on pre-training lesions to the dorsal hippocampus (DH) (Pentkowski et al., 2006). Another study, however, reported that inactivation of the DH prior to preexposure to the context impaired shock-induced contextual learning (Matus-Amat et al., 2007). It may be that contextual conditioning requires both the DH and VH (Otto and Poon, 2006) and these areas may not be so distinct from each other since they share similar circuitry (Fanselow and Dong, 2010). Although the dentate gyrus is also activated during the acute response,

is a site of neurogenesis, and receives entorhinal cortex projections, it is not involved in the consolidation of contextual memory since late-occurring protein synthesis, or genomic effects, are only found in Cornu Ammonis area 1 (CA1), which passes through both the DH and VH (Joels et al., 2007; Tsoory et al., 2007b; Vouimba et al., 2007).

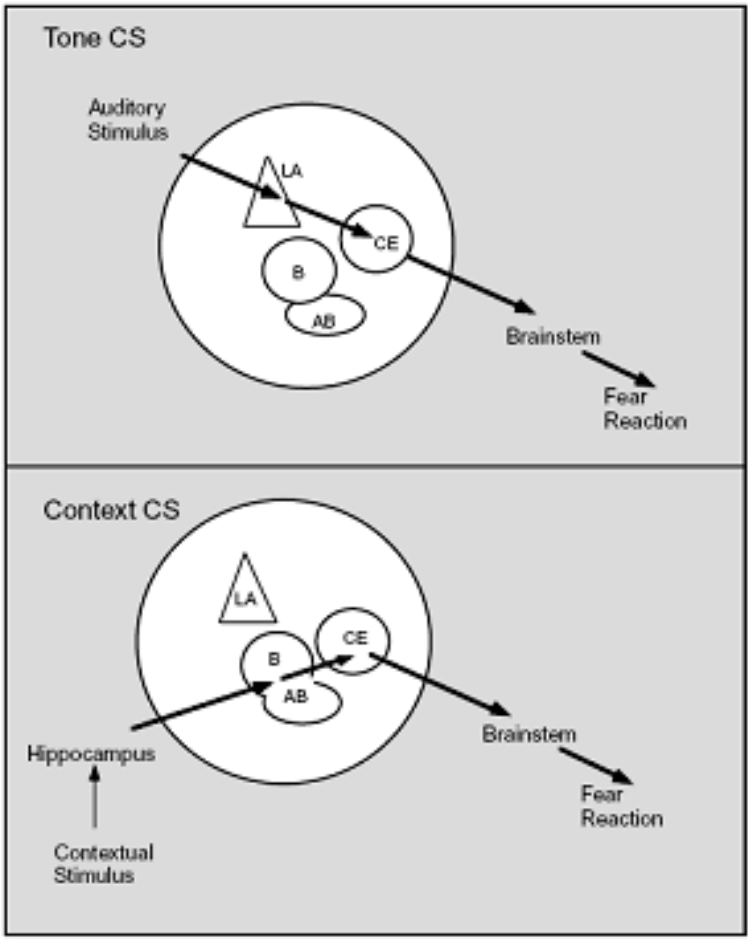


Figure 2. Critical neural substrates underlying cued and contextual conditioning. When the conditioned stimulus (CS) is a tone, projections the lateral nucleus of the amygdala (LA) and from LA to the central nucleus of the amygdala (CE) support cued conditioning. During contextual conditioning, pairing of the context (CS) and unconditioned stimulus involves the representation of the context by the hippocampus. The hippocampus and the basal (B) and accessory basal (B) nuclei of the amygdala interact and then project to CE.
From: LeDoux: ANNU REV NEUROSCI, VOL 23 (2000) 155-184.

How contextual fear memory acquisition, consolidation, and retrieval take place will be discussed now with olfaction since this mechanism is also not completely understood, especially in early life. The odor-learning circuit is complex because, across development, the interpretation of an odor can vary as ecological threats change (Wiedenmayer and Barr, 2001), the processing of odor information shifts from one mechanism to another, and young animals unable to associate an odor with a context gain this ability.

In the adult rat, odorant molecules bind to receptors within the olfactory epithelium. Axons projecting from these receptors converge in the main olfactory bulb forming glomeruli (Mombaerts et al., 1996). Mitral cells within the glomeruli send axonal projections to the piriform cortex (which is involved in odor identification), the medial amygdala (MeA), the accessory basal nucleus of the amygdala (which projects to the lateral nucleus), and the entorhinal cortex (which is the main input to the hippocampus) (Rosen, 2004; Wilson et al., 2004).

The infant rat, however, may process odor information at the level of the olfactory bulb. Sullivan (2001) found that rat pups before PN10 are able to learn and can associate the mother's odor to a milk reward. They are not, however, able to form negative associations that would cause them to avoid the mother because doing so would endanger their survival more definitely. Around PN10, cued associative learning wanes at the level of the olfactory bulb as autoexcitation between the olfactory bulb and the locus coeruleus (LC) decreases and autoinhibition emerges (Moriceau and Sullivan, 2004). Cued associative learning "switches" to the amygdala around PN7 as major nuclei subdivisions become delineated and neurogenesis begins around PN10 (Sullivan, 2001). Contextual

conditioning is not supported in the young rat until much later, weakly emerging around PN18 (Rudy and Morledge, 1994) as connections from the entorhinal cortex to the hippocampus develop (Crain et al., 1973). Young rats, those 18 days old and younger, show general deficits in contextual fear learning and perform similarly to adult animals with hippocampal damage, suggesting that the neural substrates supporting this type of learning are late to mature (Rudy, 1993).

The patterns of Fos expression in the amygdala and hippocampus differ when the US is predator odor than when it is shock in the rat; indicating that the neural circuits underlying conditioned fear responses may change depending on the US (Rosen et al., 1998; Takahashi et al., 2008). One most obvious difference in the shock versus predator odor paradigm is the unconditioned behavioral fear response which cannot be measured when using shock since the animal startles, or jumps, and does not exhibit freezing until after training. The unconditioned and conditioned fear response to predator odor can be attenuated by pre-training lesions to the MeA, basolateral amygdala (BLA), and VH with ibotenic acid (Pentkowski et al., 2006; Takahashi et al., 2007). For example, the VH is involved in the freezing response since neurotoxic lesions to this area reduce acute freezing to cat odor and footshock, while lesions to the DH do not change unconditioned freezing levels (Blanchard et al., 2005). Results from lesions to the BLA and VH with muscimol are similar to experiments with footshock, indicating that these may be areas which respond broadly to perturbation; however the MeA appears to be specific in the formation of an association of a context to predator odor (Takahashi et al., 2008). Because lesions of the BLA immediately after training and the MeA immediately before testing both result in less fear-related behavior during the test, the BLA is likely involved

in consolidation while the MeA is important for memory retrieval (Takahashi et al., 2008).

There is also evidence that suggests that the hippocampus is involved in consolidation. In one study, adult rats with hippocampal lesions displayed similar freezing behavior as sham-lesioned animals directly after shock, but they exhibited less freezing behavior than controls 24 hours later during the contextual test (Kim et al., 1993). Nevertheless, information from the context (CS) and predator odor (US) likely meet in the BLA (LeDoux, 2000). Once information is consolidated, the amygdala stores the contextual representation, which is hippocampal-dependent (Huff et al., 2005).

The context preexposure facilitation effect (CPFE) is dependent on the dorsal hippocampus (Rudy et al., 2002). Neurotoxic lesions of the DH, bilateral injections of the protein synthesis inhibitor anisomycin into the DH after preexposure, and inactivation of the DH with the gamma-aminobutyric acid $(GABA)_A$ agonist muscimol before or after preexposure, before training, or before testing all severely decrease the CPFE (Barrientos et al., 2002; Rudy et al., 2002; Matus-Amat et al., 2004). The hippocampus functions to form the conjunctive representation of the context. Although conditioning may also be achieved by learning discrete features of the context (Matus-Amat et al., 2007), blockage of the DH at different stages using the CPFE have shown that preexposure facilitates contextual learning (Rudy et al., 2004). Thus, it appears that contextual fear conditioning requires both an acquired representation of the context which can be associated with the fearful stimulus, and the hippocampus to contribute to memory storage (Barrientos et al., 2002).

IV. Norepinephrine and Fear

Several neurotransmitters have been implicated in fear learning (Davies et al., 2004). Norepinephrine (NE) is especially relevant to fear conditioning as it mediates both shock-induced contextual fear conditioning (Huff et al., 2005; Berlau and McGaugh, 2006) and the unconditioned and contextually-conditioned fear response of adult rats to cat odor (Do Monte et al., 2008). Extreme deficiencies or excesses of NE interfere with memory consolidation but moderate amounts facilitate memory (Wilson et al., 1994).

In the rat, NE is synthesized in the LC (Berridge and Waterhouse, 2003) and targets of NE release in the rat include, but are not limited to, the olfactory bulb, hippocampus, and the amygdala (Morrow et al., 2000; Joels et al., 2007; Do Monte et al., 2008). Synaptic vesicles release NE into the synaptic cleft, where it binds to α- or β-adrenergic receptors located in the post-synaptic membrane. α-receptors have the subtypes α_1 and α_2. Both subtypes are involved in smooth muscle contraction, but α_2 additionally inhibits neurotransmitter (i.e. NE) function by inactivating the molecular cascade initiated by neurotransmitter release (Davies et al., 2004) (Figure 3). Of the three subtypes of β-receptors, activation of β_1-receptors has the converse effect to α_2 and is critical to NE's contribution to long-term memory (LTM) formation (see CREB and Long-Term Memory Formation) (Figure 3).

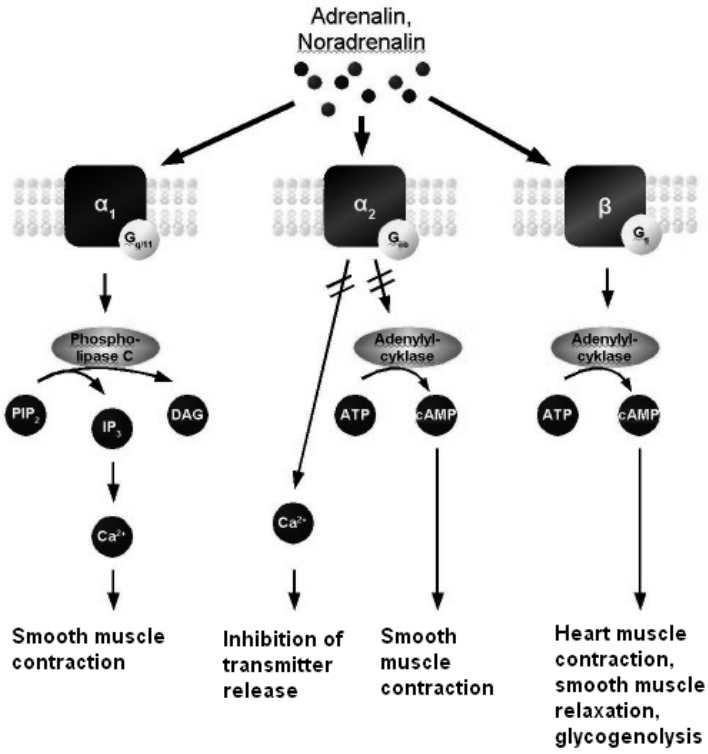


Figure 3. The mechanism of adrenergic receptors.

Studies suggest that NE also selectively modulates contextual memories (Murchison et al., 2004; Wilson et al., 2004). NE may exert its affects on the hippocampus during contextual conditioning in several ways. When an animal is faced with a severe threat, the brain is flooded with NE and general excitability is increased (Joels et al., 2007). The central release of NE globally increases arousal but can enhance memory acquisition, particularly in the BLA (Josselyn and Nguyen, 2005). Pretraining injection of the β-adrenergic receptor antagonist propranolol blocks the effects of NE on memory acquisition (Sullivan et al., 1991; Matus-Amat et al., 2007). Interaction between the amygdala and hippocampus may then facilitate the consolidation of long-term contextual memory in the BLA (Ferry et al., 1999; McGaugh and Roozendaal, 2002).

Alternatively, it may be activation of the β-adrenergic receptors in the hippocampus that is critical to NE's contribution to long-term contextual memory formation. These β-receptors have been successfully manipulated with post-training intra-CA1 infusions of propranolol, which disrupted contextual fear memory consolidation (Ji et al., 2003).

V. CREB and Long-Term Memory Formation

During learning, the induction of gene expression contributes to structural changes at the synapse, and it is this alteration of synaptic strength which encodes memories (Cooke and Bliss, 2006). Long-term potentiation (LTP), the long-lasting strengthening of synaptic signaling between two simultaneously-firing neurons, is a cellular mechanism that mediates synaptic plasticity. LTP has been studied extensively in the CA1 and is stimulated by high levels of cAMP response element binding protein (CREB) phosphorylation (Josselyn and Nguyen, 2005). CREB is a transcription factor that controls gene expression and is constitutively present in the cell nucleus without transcriptional activity (Herdegen and Leah, 1998; West et al., 2002). When neurons are stimulated, several signaling pathways mediate the phosphorylation of CREB, converting it into an active state where it regulates gene expression and participates in the cellular changes associated with LTM (Rodrigues et al., 2004; Carlezon et al., 2005; Josselyn and Nguyen, 2005).

One pathway that mediates the phosphorylation of CREB is via NE. When NE binds to β_1-receptors, a signal transduction cascade is initiated. β-receptors couple to G-proteins (G_s) that, through association with adenylyl cyclase, convert adenosine

triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP) (McLean and Harley, 2004). cAMP activates protein kinase A (PKA), which enters the cell nucleus and activates CREB (McLean and Harley, 2004). Phosphorylated CREB (pCREB) then binds to DNA through cAMP response elements (CRE) and turns on the transcription of genes leading to the expression of new proteins from mRNA (Thonberg et al., 2002).

pCREB is expressed in hippocampal CA1 cells after contextual fear conditioning to shock in adult mice (Sindreu et al., 2007) and after stress in PN18 and adult rats (Chen et al., 2006). CREB activity also occurs in early ontogeny, with levels of pCREB in the hippocampus changing in a cell-specific way during the first weeks of life in rats (Bender et al., 2001). In rat pups, pCREB mediates cued odor learning in the olfactory bulb (McLean and Harley, 2004). The relationships between pCREB, NE, and the emergence of contextual conditioning are unknown.

VI. Proposed Model

We postulate that the fear response changes throughout ontogeny and that some of these changes are associated with the stimulus, the environment, or a combination of these factors. A better understanding of the unlearned fear response and the conditions under which learning occurs can be used to model human trauma in early life. We established that contextual fear conditioning is mediated by the hippocampus and postulate that it is modulated by NE, as shown in Figure 4. After odor information converges in the olfactory bulb, axonal projections relay this information to the piriform cortex, the amygdala, and the entorhinal cortex. The piriform cortex identifies cat odor as

emotionally-relevant and NE is released from ascending axon bundles projecting from the LC to the amygdala and hippocampus. Relevant to our model of contextual learning, levels of pCREB increase in the hippocampus and support the formation of LTM.

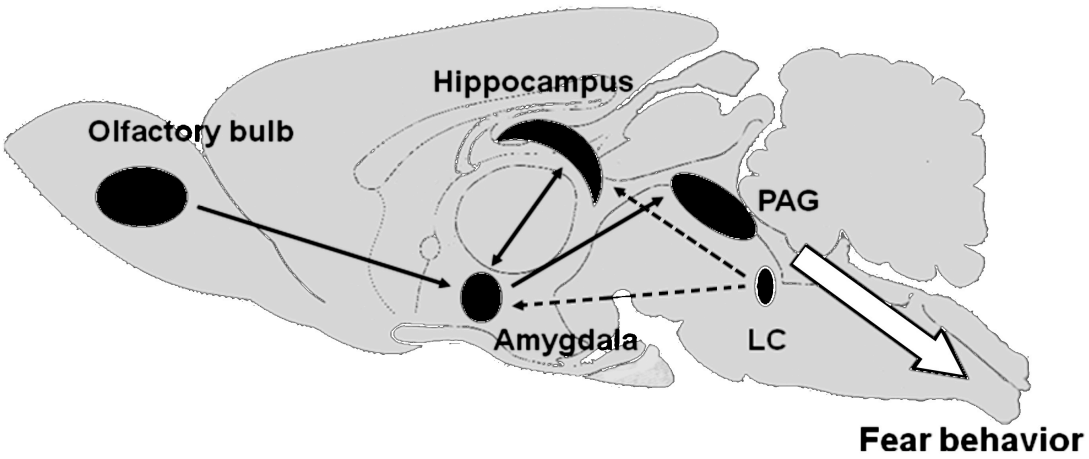


Figure 4. Proposed model by which norepinephrine modulates contextual fear learning in young animals exposed to predator odor. NE release from ascending LC axon bundles may influence both the amygdala and hippocampus. We expect that the dual action of NE on the hippocampus via β-receptors present in the amygdala and in the hippocampus itself increases pCREB levels when the animal learns about the context in which a predator odor was encountered. Dotted arrows indicate cascades activated during exposure to predator odor. Solid arrows indicate NE's action on the amygdala and hippocampus.

Specific Aims

In humans, traumatic events during adulthood can induce long-lasting fear memories. However, traumas often occur early in life and can have profound effects on the emergence of psychopathologies such as major depression and anxiety disorders (Teicher et al., 2003). Although fear conditioning is only one of several mechanisms that are relevant to human psychopathology after trauma, a better understanding of fear memory formation in early development is critical in building a translational model of

childhood trauma. Animal models can be used to explore the causal relationships between aversive events and psychopathologies such as post-traumatic stress disorder (PTSD) (Wiedenmayer, 2004). However, most current investigations use adult animals in paradigms that employ stimuli with little ecological relevance in limited environmental contexts.

The role of assorted ecologically-relevant stimuli in different spatial contexts at diverse ages on the unlearned fear response is unclear. Further, when and how young animals learn about fear-inducing stimuli has also not been thoroughly investigated. That is, the mechanism by which contextual fear conditioning occurs in young animals trained with ecologically-relevant stimuli is not known. By understanding the basis of the fear response and fear learning in young organisms and the variables that affect this learning, a model of learning in young animals will be created that can be used to study the mechanisms of early fear conditioning. It has been noted that there currently exists no agreed-upon model for PTSD (Tsoory et al., 2007b). Ecologically-relevant stimuli may be used to better model anxiety disorders such as PTSD induced early in life, leading to improved intervention and treatment.

The same stimulus has not previously been used to induce unlearned fear across the rat's lifespan. In addition, very little is known about early contextual fear learning to a stimulus known to produce learning in adulthood. Thus, the purpose of my dissertation is to first understand the experimental parameters of unlearned and learned fear to predator odor, and then to test the mechanisms underlying contextual fear conditioning to predator odor in early life. We hypothesize *(1) that unlearned fear responses of rats change as a function of the stimulus, the environment, and age; (2) that contextual fear learning*

occurs early in life in response to ecologically-relevant stimuli; and (3) that the noradrenergic system is involved in hippocampal-dependent contextual fear learning. The ecologically-relevant experimental paradigm that we propose will allow us to test specific predictions about contextual and stimulus variables that modulate the fear response in an age-specific way. The contextual fear learning paradigm that we develop for juvenile rats will allow us to first model early trauma, and then manipulate it using pharmacological methods. To test these hypotheses, the following specific aims are proposed:

Aim 1: To test the hypothesis that the unlearned fear responses of rats change as a function of the stimulus, the environment, and age. To this end, experiments are designed to test if differences in unlearned fear responses are induced using ecologically relevant odor cues (cat urine, cat fur) or a control odor (lavender oil) in a small and large chamber. The groups used are infant (PN14), juvenile (PN26), adolescent (diestrus females PN35; males PN45), and adult (PN>90) rats. Fear-related behaviors measured are freezing and risk-assessment. We predict that all ages will display fear-related behavior to cat odors but that younger animals will freeze while older animals will increase risk-assessment behaviors. We further predict that animals will increase fear-related behavior to cat fur compared to cat urine and the control odor. Lastly, we expect that animals exposed to cat odors will respond with heightened levels of freezing behavior in the small chamber and heightened levels of risk-assessment in the large chamber.

Aim 2: To develop a contextual fear learning paradigm utilizing ecologically relevant stimuli which can be used in young rats. To this end, infant and juvenile rats are preexposed to the context, exposed to either cat odor or the control odor the following day (training), and returned to the chamber for contextual testing 24 hours later. A hide box is provided on all days. Behavior measured during odor exposure and the contextual test is freezing. We predicted that both infant and juvenile rats would recognize and respond to cat odor but that only juvenile rats would form contextual fear memories.

Aim 3: To test the hypotheses that the noradrenergic system is involved in contextual fear learning in juvenile but not infant rats and that this contextual learning is hippocampal-dependent. To this end, experiments are designed to test the effects of systemic injections of the β-adrenergic receptor antagonist propranolol at two different time periods on the unlearned fear response and contextual learning in juvenile rats exposed to either cat odor or the control odor. We propose that β-adrenergic blockade would block the positive effects of NE on behavioral responsivity and contextual learning to threat. Freezing behavior is measured during the odor exposure and the contextual test. We predict that during odor exposure juvenile rats previously injected with propranolol will freeze less to the cat odor than rats previously injected with saline. We further predict that these same propranolol-injected rats will freeze less than their saline-injected counterparts during the contextual test. In animals injected with propranolol after exposure to the cat odor, we also expect a decrease in the learned fear response. In order to rule out peripheral effects of NE on the contextual learning, we will systemically inject juvenile rats with the β-adrenergic receptor antagonist nadolol, which does not cross the

blood-brain barrier. Additionally, pCREB will be measured in the DH and VH after training in rats injected with propranolol or saline. We expect increased pCREB levels to be present in the hippocampi of juvenile rats injected with saline and exposed to the cat odor compared to all other groups. Lastly, we will compare CREB and pCREB levels in juvenile rats to infant rats, which are unable to achieve contextual learning.

Chapter 2: Effects of the Stimulus and Chamber Size on Unlearned Fear across Development

1. Introduction

The ability of an organism to innately respond appropriately to a severe threat has ultimate survival advantages (Kavaliers and Choleris, 2001). For example, adult rats respond to cat odors by engaging in increased freezing and risk-assessment behaviors (Blanchard et al., 1990; Zangrossi and File, 1992; Dielenberg and McGregor, 2001). The rat may avoid detection by the cat by freezing, while various risk-assessment behaviors such as stretch-attends or flatback approaches allow the rat to monitor a potential threat. Risk-assessment may be a more adaptive response to predator stimuli than freezing in the adult rat, particularly under conditions of low danger when time and energy can be better spent in activities such as caring for young or foraging (Klein et al., 1994; Blanchard et al., 2003a). However, little is known about how developing animals respond to various predator odor cues (but see (Muller-Schwarze, 1972; Lyons and Banks, 1982; Moriceau et al., 2004). Specifically, it remains to be determined which cues are the most effective at eliciting a fear response at different ages and what these cues may represent for these age groups.

Prey response to a predator is also dependent upon the amount of space which separates them, according to Fanselow and Lester's predator imminence model (1988). Predator/prey interactions model fear and anxiety depending on whether or not a threat is immediate or anticipated, and this threat level can be manipulated by changing the type of stimulus used and the space available in the experimental chamber (Blanchard and Blanchard, 1990). If the predator is in close proximity, the prey has little opportunity for

escape and thus primarily freezes to decrease conspicuousness. If there exists a greater distance between predator and prey, the prey may engage in risk-assessment or avoidance behaviors. Therefore, space confines affect an animal's behavior by increasing its perceived predation risk due to a lack of defense options and reducing the potential for different coping behaviors (i.e. risk-assessment) to emerge (Ward et al., 1997).

The type of stimuli and its proximity interact to produce a signal of danger, however the exact conditions which direct behaviors in one direction or another are not completely understood (de Oca et al., 2007). Most experimental paradigms using rats and predator odors have more than likely exaggerated fear learning due to the intensity of the stimulus used and small spatial confines of the apparatus. Additionally, unlearned fear behavior to a predator should vary with the age of the respondent. To test the hypothesis that behavioral responses of rats change as a function of these factors, infant, juvenile, adolescent, and adult rats were exposed to either cat fur, cat urine, or a control odor in a small or large chamber. It was hypothesized that all ages would display fear-related behavior to cat odors since these stimuli have been found to be effective in inducing behavioral changes in adult and juvenile rats (McGregor et al., 2002; Blanchard et al., 2003b; Hubbard et al., 2004).

However, it was expected that different age groups would use different strategies to cope with threat. Younger animals, unable to flee as effectively as older animals, were expected to prefer freezing over risk-assessment while older animals should display increased risk-assessment over freezing. It was expected that animals would display an increase in fear-related behavior during exposure to cat fur compared to cat urine and the control odor since cat fur odor may signify a higher level of threat and the actual presence

of the predator than a scatological odor cue that may signify that the predator was historically present (Blanchard and Blanchard, 1989). Lastly, based on the predator imminence model (Fanselow and Lester, 1988), it was also predicted that animals exposed to cat odors would respond with heightened levels of freezing behavior in the small chamber and heightened levels of risk-assessment in the large chamber.

2. Materials and Methods

2.1 Subjects

All experiments used wild-type Long-Evans hooded rats bred in the animal care facility of Columbia University and housed under standard laboratory conditions. Cages were monitored daily in the morning and evening for the presence of newborn pups and the date of birth was considered as post-natal day (PN) 0. Rats were separated from the dam on PN23 but remained with littermates in the same cage. Adolescent and adult rats were kept in cages with one or two same-sex littermates. Rats of four different ages – infant (PN14), juvenile (PN26), adolescent (male: PN35, female: PN45), and adult (PN90-100) were used. For all behavioral experiments both female and male animals were used since no sex differences in threat-induced freezing in infant and juvenile rats have been observed (Wiedenmayer and Barr, 1998). The infant animals were odor-exposed on PN14 and the juvenile animals on PN26. For the adolescent group of animals, 35 day-old females in diestrus and 45 day-old males were used since adolescence begins earlier in female rats than male rats (Coe et al., 1981). Estrus cycle was determined by vaginal swab cytology. Each animal was tested in only one experimental condition.

2.2 Apparatus

In order to test odor stimuli for different ages of rats, an acrylic box with a hinged top lid and internal width and height of 15.25 x 20.25 cm was used (Figure 5). The box contained a movable, solid divider that was adjusted depending on the age of the rat and contextual condition. At each age, both a small chamber (approximately one body length) and a large chamber (3 times the approximate body length) were used. For infant rats, the total length of the small chamber was 7.6 cm and 22.9 cm for the large chamber; juvenile

rats, 10.2 cm and 30.5 cm; adolescent rats, 15.2 cm and 45.7 cm; and for adult rats, 22.9 cm and 71.1 cm. The chamber was cleaned with 70 % isopropyl alcohol before each use and paper towel was taped to the floor of the chamber. In the large testing chamber condition, a 3 (across length) x 2 (across width) grid was drawn on the paper towel in order to measure proximity and activity.

2.3 Odor Stimuli

The odor stimulus was delivered via an odor box that affixed over the odor port on the outside of the apparatus (see Figure 5). The stimulus consisted of cat fur, cat urine, or approximately 0.1mL of pure essential lavender oil (Imperial Drug & Spice Corp., West New York, NJ) presented on Kimwipe. The cat fur odor cue was a piece of polyester Berber fleece that had been used as a bed for several different cats in the animal care facility for approximately 12 months. The fleece was cut into squares of approximately 5 x 7.5 cm and stored at -80 degrees Celsius. The cat urine stimulus was urine collected from a 16 year-old spayed female domestic cat. Urine was stored at -80 degrees Celsius and approximately 2 mL presented on a Kimwipe during exposure. Lavender oil was used as the control since pilot studies showed that rats do not display fear behaviors to this odorant.

2.4 Behavioral Measures

Two commonly studied kinds of fear responses were quantified: freezing and risk assessment. Freezing, the absence of all movement except for what is necessary for respiration, is a common fear response of adult rats encountering a cat or cat odor in the

laboratory (Blanchard and Blanchard, 1971; Hubbard et al., 2004) and has been seen at younger ages (Wiedenmayer et al., 2005). Risk-assessment was quantified as the sum of stretch attend (head extended toward stimulus with low, flat back), flatback approach (slow movement towards stimulus with flat back), and head-up (head movement with nose in the air) behaviors (described by (Dielenberg and McGregor, 2001; Hubbard et al., 2004). Behavior was recorded using a Panasonic PV-GS300 digital video camcorder mounted next to the chamber. Videos were transferred to a computer hard-drive then analyzed with The Observer XT 7.0 (Noldus Information Technology) and screened by an observer unaware of the experimental conditions. All behaviors were quantified for duration.

2.5 Procedure

To reduce handling-associated stress, the experimenter picked up and briefly (~120 sec) held each juvenile, adolescent, and adult animal on the 2 days prior to odor exposure to reduce handling-associated stress (Levine, 2005). Under dim light conditions in the testing room, each rat was exposed to one of the three stimuli in either a small or large chamber. During exposure, a single animal was placed in the apparatus and allowed to habituate for two minutes. One stimulus condition was then presented via the vent port to the animal in the chamber for three minutes. The order of stimulus conditions, chamber size, and the sex of animals were counterbalanced. Animals were separated from littermates after exposure until all animals for the session had been run. In order to conserve body heat, infant animals were placed on a heating pad between sessions and prior to returning to the home cage.

2.6 Data Analysis

A three-way multivariate analysis of variance (MANOVA) was conducted to determine the effect of the three types of stimuli (cat fur, cat urine and control odor), the two sizes of chambers (small and large), and four ages (infant, juvenile, adolescent, and adult) on the two dependent variables, freezing and risk-assessment. Analyses of variances (ANOVAs) on the dependent variables were conducted as follow-up tests to the MANOVA. Using the Bonferroni method, each ANOVA was tested at the .025 level (.05 divided by the number of dependent variables) to control for Type I error. When a significant interaction was found, post hoc tests were conducted to evaluate the pairwise differences among the means with α set at .025. All statistical tests were performed using SPSS (v. 17 for Windows, Chicago, IL) and all graphs were generated using GraphPad Prism (v. 5 for Windows, San Diego, CA).

3. Results

3.1 Effects of Age

The 4 Age X 3 Stimulus X 2 Chamber Size MANOVA indicated a significant 4 X 3 interaction between age and the stimulus, Wilks's $\Lambda = 0.74$, $F_{(12, 238)} = 3.22$, $p < .001$ (observed power = .99). There was no effect of chamber size on the dependent variables of freezing (Figure 6) and risk-assessment (Figure 7), Wilks's $\Lambda = 0.98$, $F_{(2, 119)} = 1.24$, $p = .29$ (observed power = .27).

As predicted, differences were found in freezing behavior (ANOVA significant 4 X 3 interaction: $F_{(6, 120)} = 4.39$, $p < .001$; observed power = .98) among the four ages in response to the three different stimuli (Figure 8). Contrary to our expectations, however,

there were no statistical differences between ages on the dependent measure of risk-assessment ($F_{(6, 120)} = 2.32$, $p = .04$; observed power = .79) to the various stimuli (Figure 9). Infants froze more to cat fur than did juveniles ($p < .01$), adolescents ($p < .001$), or adults ($p < .001$). Infants also displayed increased freezing behavior to cat urine than adolescents ($p < .001$) and adults ($p < .025$). Juveniles froze more to cat fur than adolescents ($p < .025$) and adults ($p < .01$). There were no differences in freezing between adolescents and adults. In sum, the youngest two groups of animals froze more to cat odor cues than the older two groups but the older animals did not engage in more risk-assessment than the younger animals.

3.2 Effects of the Stimulus

It was predicted that animals would display increased fear-related behavior to cat fur over all other stimuli. As reported above, this prediction was supported for the measure of freezing but not for the measure of risk-assessment. Although all ages of animals froze more during exposure to cat fur compared to the control odor (infants and juveniles: $p < .001$; adolescents and adults: $p < .025$), the youngest two ages additionally froze more during exposure to cat urine compared to the control odor (infants: $p < .001$; juveniles: $p < .025$) (Figure 10). Surprisingly, adults were the only group that did not freeze more to cat fur than cat urine (infants: $p < .001$; juveniles: $p < .01$; adolescents: $p < .025$) (Figure 10).

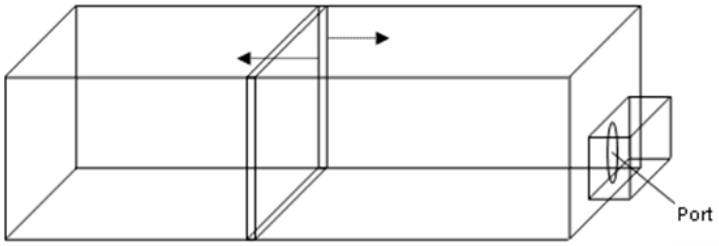


Figure 5. Odor conditioning apparatus. Rats are exposed to cat cues that are presented at the odor port. The size of the chamber is adjusted by a divider wall.

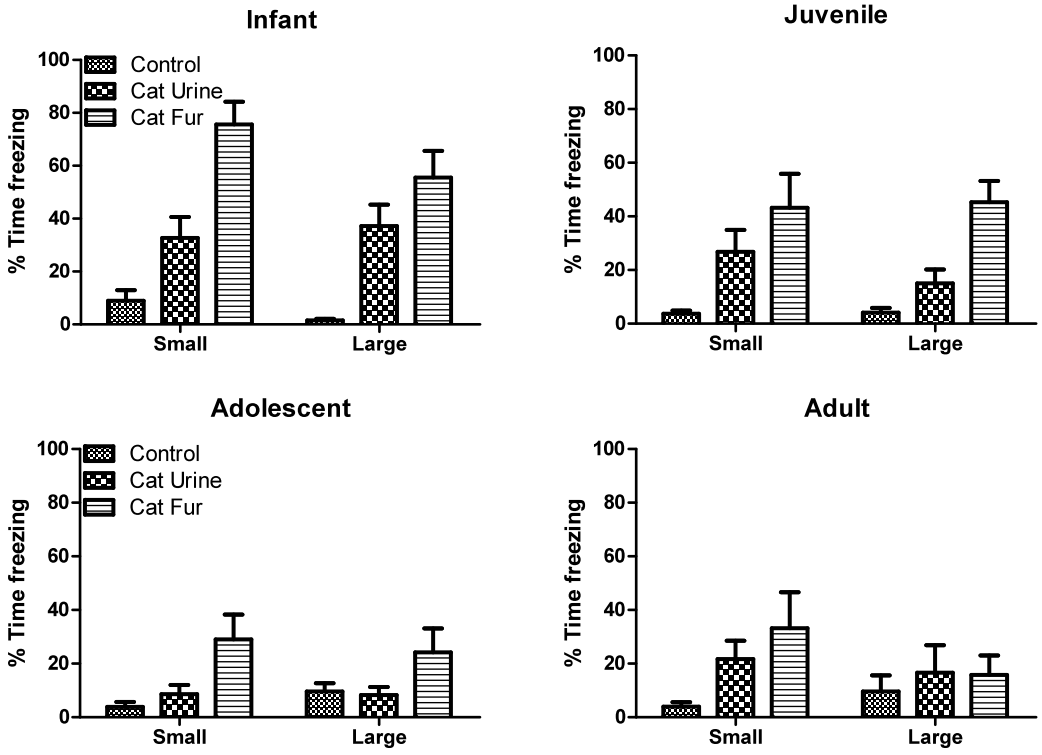


Figure 6. Freezing behavior is unaffected by chamber size. Mean and SEM percent time freezing during odor exposure ($N = 6$ per group).

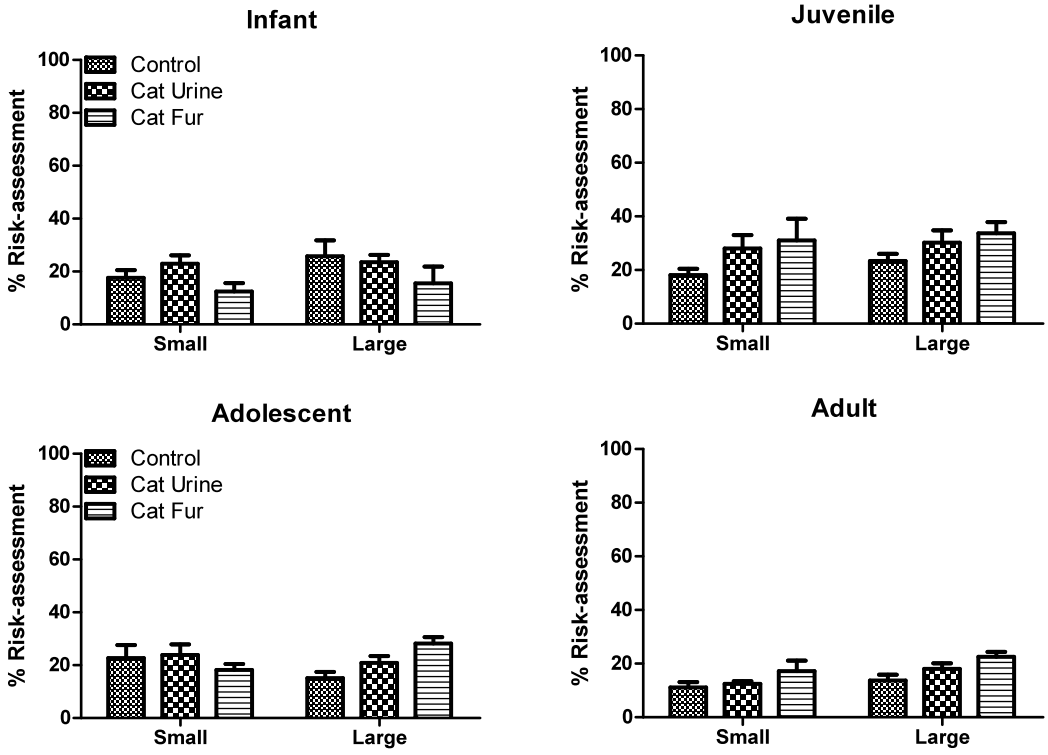


Figure 7. Risk-assessment does not change with chamber size. Mean and SEM percent time engaging in risk-assessment behaviors during odor exposure ($N = 6$ per group).

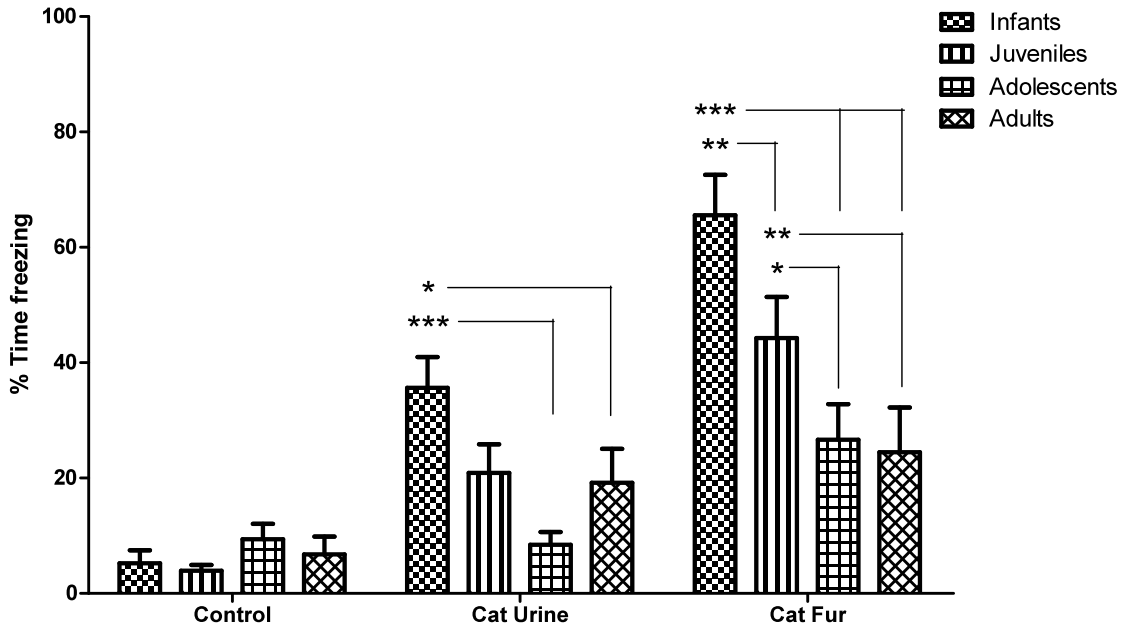


Figure 8. Cat fur is a highly effective fear-inducing stimulus across the lifespan. Mean and SEM percent time freezing during odor exposure. For the effect of age, infants and juveniles displayed increased levels of freezing to cat odor cues compared to adolescents and adults. Because there was no effect of chamber size on freezing behavior, data for each stimulus condition are collapsed over this factor (*** $p < .001$, ** $p < .01$, * $p < .025$; $N = 12$ per group).

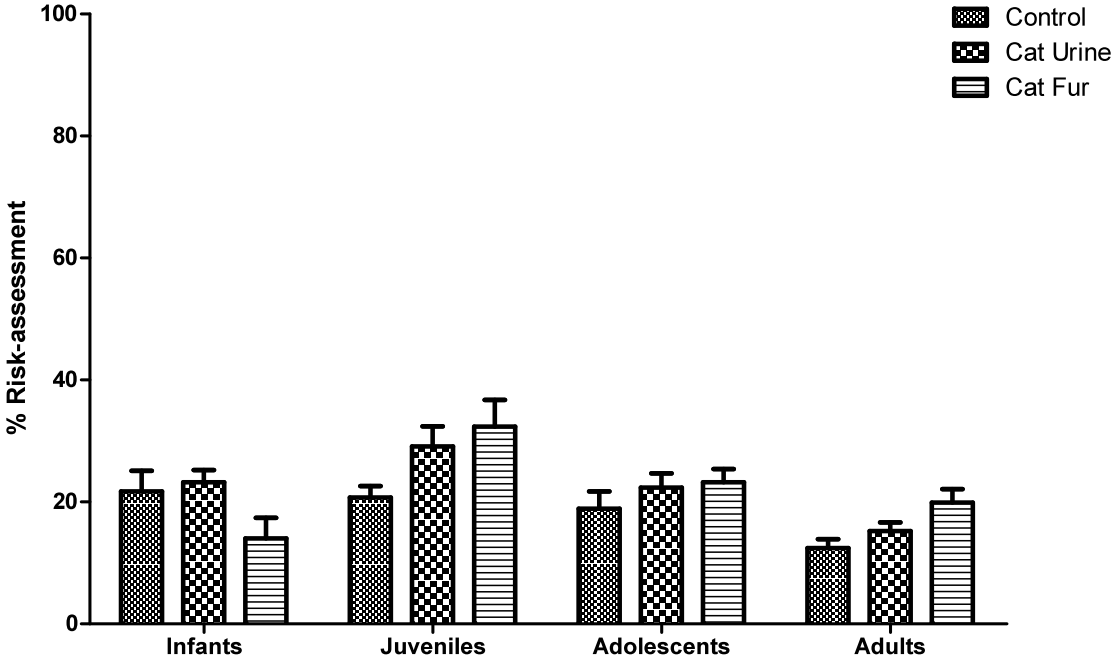


Figure 9. Risk-assessment does not differ across age or stimulus. Mean and SEM percent time engaging in risk-assessment behaviors during odor exposure. Data for each stimulus condition are collapsed over chamber size ($N = 12$ per group).

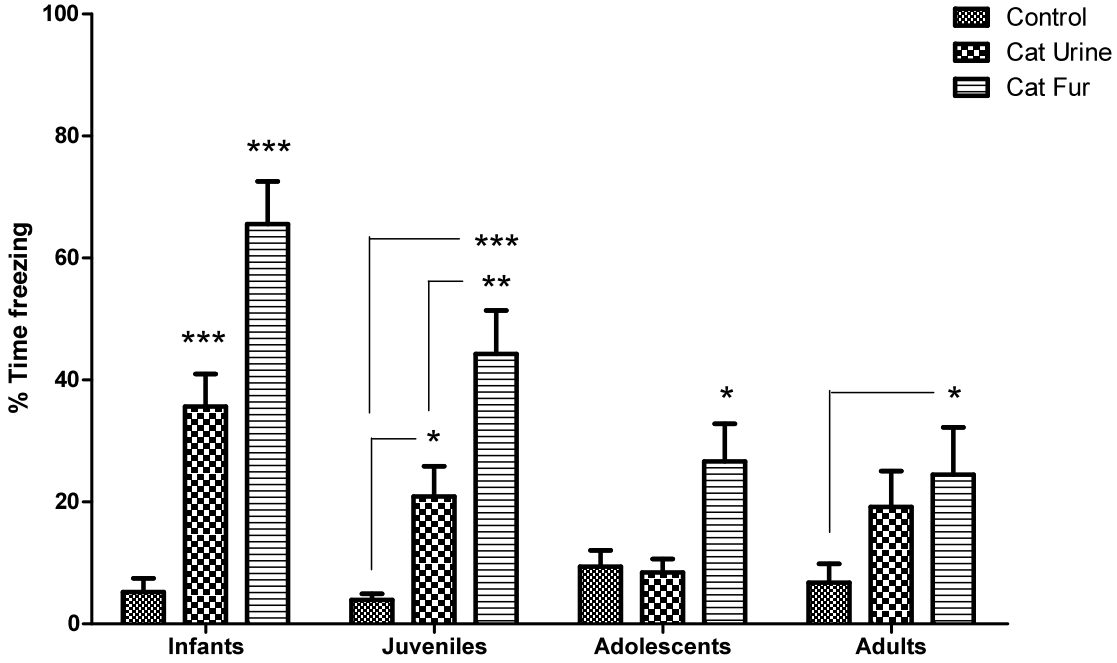


Figure 10. Fear responsivity to different cat odor cues changes throughout life. Mean and SEM percent time freezing during odor exposure. Cat urine additionally increased freezing in younger animals compared to the control odor (*** $p < .001$, ** $p < .01$, * $p < .025$; $N = 12$ per group).

4. Discussion

Unlearned fear behavior in rats changed as a function of age and the stimulus, but not of chamber size, partially supporting the hypothesis. Though all ages displayed fear-related behavior to cat odors, differences were reflected only in freezing behavior and not, as expected, risk-assessment. High levels of freezing behavior in infants and juveniles support the hypothesis that younger animals utilize this strategy preferentially over risk-assessment.

The mechanisms mediating unlearned fear responses to ecologically-relevant stimuli have likely been shaped by evolutionary processes, as adaptive behavioral responses are expected to be different across the lifespan when ecological threats change (Wiedenmayer, 2009), physical abilities develop, and home-ranges expand. For example, neonatal rats are naturally under less predation risk when sheltered in a burrow. On the other hand, when attacked, they may not have the physical coordination to flee. Both inexperience and physical immaturity could mean that heightened fear-related behavior (i.e. freezing) is both appropriate and adaptive behavior for such young animals. Around weaning, rats may naturally engage in more risk-assessment behavior if separated from the dam and their littermates. Peri-adolescent rats, who are hyperactive under naturalistic conditions, may display heightened exploratory and risk-assessment behaviors (Spear and Brake, 1983).

That animals unanimously responded with fear to cat fur compared to the control odor was expected, based on findings with adult rats (for review, see (Takahashi et al., 2008). However, increased fear to cat urine than the control odor in infants and juveniles is a novel finding. This finding does not entirely conform to Blanchard et al.'s (2003b)

suggestion that scatological cues are less threatening than cat fur, especially as adult animals did not respond to cat urine significantly less than cat fur. A likely explanation for high levels of freezing to cat urine in young animals is that these age groups possess limited defensive options to cope with threat and so may respond with freezing to all predator stimuli. For adults, this result was likely an artifact of low freezing to cat fur (Li et al., 2004; Takahashi et al., 2007).

The sizes of the chamber had no effect on either freezing or risk-assessment in this study. It may be that the chamber sizes used were not dissimilar enough to induce differences in unlearned fear-related behavior.

Chapter 3: Norepinephrine Mediates Contextual Fear Learning and Hippocampal pCREB in the Juvenile Rat in Response to Predator Odor

1. Introduction

Contextual fear conditioning can be adaptive, under natural conditions, as animals that are able to remember specific locations in which predator odors were encountered could increase their survival and subsequent fitness by treating those areas with caution in the future. Indeed, live cat and natural cat fur or skin odors have been found to be strong unconditioned stimuli for contextual fear conditioning in adult rats – altering their behaviors in the conditioned context for long periods of time (Blanchard and Blanchard, 1989; Blanchard et al., 2001). However, fear conditioning in early life differs from fear learning in adulthood as animals fail to form shock-induced contextual fear memories until postnatal day 18 (PN18) (Rudy and Morledge, 1994). It is not clear at what age young animals form contextual fear memories of predator odor. In one of the few experiments using young rats (P18, 26, and 38), a cat-rubbed or odorless cloth-covered block was placed in the experimental chamber during training and animals were returned to the context containing the odorless cloth-covered block during testing (Hubbard et al., 2004). This experiment showed that juvenile rats could learn from predator odor cues but it remained unclear if this learning was contextual or cued as the block likely was a cue.

Young animals cannot be contextually fear-conditioned to shock because the neural substrates that underlie this ability may be late to mature (Rudy, 1993; Rudy and Morledge, 1994). Two areas, the amygdala and the hippocampus, are necessary for contextual fear conditioning in adult animals (LeDoux, 2000). The amygdala is functional in early life as infant rats demonstrate amygdala-dependent cued conditioning

by PN10 (Sullivan et al., 2000b). It is likely the hippocampus that is immature in infant rats but whether it mediates contextual learning early in life has not been tested.

Fear conditioning in general requires memory acquisition, consolidation, and retrieval of the association. Although the amygdalar nuclei activated during each of these phases have been defined (Takahashi et al., 2008), the role of the hippocampus in contextual fear conditioning is less clear. The ventral hippocampus (VH) is involved in acquisition of memories of both shock and predator odor (Fendt and Fanselow, 1999; Bast et al., 2001; Rudy and Matus-Amat, 2005; Pentkowski et al., 2006). There are, however, discrepancies between reports regarding the role of the dorsal hippocampus (DH). In shock paradigms, the DH facilitates contextual memory acquisition but not consolidation (Schenberg and Oliveira, 2008) and consolidation but not acquisition (Kim et al., 1993; Frankland et al., 1998). Likewise, some studies report that the DH is involved in context discrimination and the acquisition of the contextual representation during preexposure but not exposure (Frankland et al., 1998; Matus-Amat et al., 2007).

Others propose that the DH is critical in contextual conditioning only if the context is overshadowed by another, more salient, conditioned stimulus (Phillips and LeDoux, 1994) or if the unconditioned stimulus (US) is weak (Quinn et al., 2008). Taken together, these studies suggest that the hippocampus is involved in acquisition and consolidation under some conditions, but the specific areas that mediate each of these processes remain under debate. The latest research suggests that the DH and VH may not be so distinct from each other since they share similar circuitry (Fanselow and Dong, 2010) and post-stress genomic effects are found in layer CA1, which passes through both the DH and VH (Joels et al., 2007; Tsoory et al., 2007b; Vouimba et al., 2007).

Differences in DH function in contextual conditioning may be US-dependent. DH lesioning does not affect contextual conditioning to predator odor as it does with shock (Pentkowski et al., 2006).

Several neurotransmitter systems have been implicated in fear learning (Davies et al., 2004; Benarroch, 2009). In rats, the central release of norepinephrine (NE) and its subsequent effect on β-adrenergic receptors within the brain plays a critical role in unlearned and conditioned fear (Do Monte et al., 2008). NE has been implicated in memory acquisition, consolidation, and retrieval – targeting receptors in the olfactory bulb, hippocampus, and the amygdala (McGaugh and Roozendaal, 2002; Murchison et al., 2004; Joels et al., 2007; Do Monte et al., 2008). Additional work has shown that NE selectively modulates contextual memories (Murchison et al., 2004; Wilson et al., 2004). When an animal is faced with a severe threat, like a predator, NE is released from the locus coeruleus (LC) and increases general neuronal excitability (Joels et al., 2007). As a consequence, the animal focuses attention on gathering sensory information about the stimulus and context (Berridge and Waterhouse, 2003; Takahashi et al., 2008). During this aroused state, NE could be involved in contextual fear acquisition.

Memory consolidation, occurring within a few minutes to a few hours after training, requires protein synthesis to support new or reconfigured synaptic connections (Dudai, 2004). It is known that NE binds to β-adrenergic receptors in the amygdala, activating projections to the CA1 layer of the hippocampus, which facilitates the consolidation of long-term contextual memory in the BLA (McGaugh and Roozendaal, 2002; Kogan and Richter-Levin, 2008). However, activation of the β-adrenergic receptors in the hippocampus could also be critical to long-term contextual fear memory. Indeed,

blocking these receptors in CA1 disrupts contextual fear memory consolidation (Ji et al., 2003). NE is one factor that promotes protein synthesis, and if blocking its effects prevents protein synthesis in the hippocampus then this would be evidence for the role of the hippocampus in NE-mediated contextual fear memory.

When NE binds to β-adrenergic receptors, a signal transduction cascade triggers the second messenger cyclic adenosine monophosphate pathway (cAMP) that activates protein kinase A (PKA) (McLean and Harley, 2004). PKA enters the cell nucleus and turns on the transcription factor cAMP response element binding protein (CREB) (McLean and Harley, 2004). CREB is present in the cell nucleus without being transcriptionally active (Herdegen and Leah, 1998; West et al., 2002). In its active state, phosphorylated CREB (pCREB) binds to DNA through cAMP response elements (CRE), turns on the transcription of genes leading to the expression of new proteins (Thonberg et al., 2002). These proteins are important for the cellular changes associated with LTM (Rodrigues et al., 2004; Carlezon et al., 2005; Josselyn and Nguyen, 2005). pCREB is elevated in hippocampal CA1 cells after contextual fear conditioning to shock in adult mice (Sindreu et al., 2007).

pCREB is also important for learning in young animals. For example, in rat pups, pCREB mediates cued odor learning in the olfactory bulb when an odor is paired with stroking (McLean et al., 1999). However, how hippocampal CREB is related to long-term contextual fear learning across early development has not yet been investigated. pCREB levels have been successfully reduced by blocking the actions of NE with the β-adrenergic antagonist propranolol. Although propranolol blocks contextual fear memory when injected systemically or into the CA1 region of the hippocampus in both shock and

predator odor paradigms (Ji et al., 2003; Do Monte et al., 2008), the consequences of propranolol administration on hippocampal pCREB remain unknown.

The goal of the present study was to examine the mechanisms underlying predator-induced contextual fear learning in early life. To evaluate these mechanisms, the role of NE in the unlearned and learned fear response and the effects of NE blockade on hippocampal pCREB were assessed.

2. Materials and Methods

2.1 Subjects

All experiments used wild-type Long-Evans hooded rats bred in the animal care facility of Columbia University and housed under standard laboratory conditions. Cages were monitored daily in the morning and evening for the presence of newborn pups and the date of birth was considered as day 0. Rats were weaned on PN23 with littermates were kept together in the same cage. The infant animals were trained on PN14 and the juvenile animals PN26. For all behavioral experiments both female and male animals were used since there are no sex differences in threat-induced freezing in infant and juvenile rats (Wiedenmayer and Barr, 1998).

2.2 Apparatus

In order to test odor stimuli for different ages of rats, an acrylic box with a hinged top lid and internal width and height of 15.25 x 20.25 cm was used (Figure 11A). The box contained a movable divider with a small opening that allowed access to the hide box. The total length of the apparatus was 18 cm for infants and 36 cm for juveniles, 3 times the approximate lengths of animals at these ages. The hide box accounted for the furthest third of the total distance from the odor port, at the opposite end of the apparatus (Figure 11B). Since volatility is not a feature of cat fur odor (Dielenberg and McGregor, 1999), the odor port was at the opposite end of the chamber to create a gradient of odor intensity across the apparatus. The size of the hide box was 6 x 4 x 4 cm for infants and 12 x 4 x 4 cm for juveniles. The chamber and hide box were cleaned with alcohol before each use.

2.3 Odor Stimuli

The odor stimulus was delivered via an odor box that affixed over the odor port on the outside of the apparatus (see Figure 11A). The stimulus consisted of either the cat odor or approximately 0.1mL of pure essential lavender oil (Imperial Drug & Spice Corp., West New York, NJ) presented on kimwipe. Pilot work revealed that both infant and juvenile rats would freeze to a cat odor cue of polyester Berber fleece that had been used as a bed for cats in the colony for approximately 12 months and contained a mix of cat fur, skin oils, saliva, and waste materials. The fleece was cut into squares of approximately 5 x 7.5 cm and stored at -80 degrees Celsius. Lavender oil was used as the control since pilot studies showed that rats do not display fear behaviors to this odor and to reduce novelty effects.

2.4 Behavioral Measures

In response to cat odor, rat's commonly observed pattern of behavior is to terminate exploratory and grooming behaviors, retreat to a location of lower danger (i.e., a hide box) and then engage in freezing and 'head out' behavior in order to decrease conspicuousness and supervise the threat, respectively (Blanchard and Blanchard, 1989; Dielenberg and McGregor, 2001). Such an escape may be more analogous to natural environmental conditions since rodents are thigmotaxic and tend to operate from a defensible position (Dielenberg et al., 1999; Whishaw et al., 2006). Without a place to hide, the rat may engage in a variety of other behaviors such as exploration as they seek out a hiding place. In all experiments a hide box was used and freezing behavior was measured. Freezing, or complete immobility, is a common fear response of adult rats

encountering a cat or cat odor in the laboratory (Blanchard and Blanchard, 1971; Hubbard et al., 2004). Although predator exposure induces other behaviors (i.e. avoidance, risk-assessment) freezing behavior is the most commonly used measure of fear.

Behavior was recorded by a Panasonic PV-GS300 digital video camcorder mounted next to the chamber. Videos were transferred to a computer hard-drive then analyzed with The Observer XT (Noldus Information Technology) and screened by an observer unaware of the experimental conditions. Freezing behavior was quantified for duration.

2.5 Procedure

Experiment 1

Lights in the behavioral testing room were dimmed in order to reduce light-enhanced aversion. A single animal was placed in the apparatus and preexposed to the context for five minutes on the day before training. After preexposure, the tail was marked with permanent ink. During training, each animal was placed in the apparatus and allowed to habituate for two minutes with no odor stimulus present. One stimulus condition was then presented via the odor port to the animal in the chamber for five minutes. Delay conditioning, where the CS overlaps the US, was used since it is most effective for contextual fear conditioning (Burman and Gewirtz, 2004). It is also ecologically appropriate since an animal should learn to avoid the context where it experienced the predator instead of the location it was in long before or after a predator encounter as would be the case with backward or trace conditioning.

The order of stimulus conditions and the sex of animals were counterbalanced across days. Animals were separated from littermates after exposure until all animals for the session had been run but were never alone except in the testing chamber. In order to conserve body heat, infant animals were placed on a heating pad between session and prior to returning to the home cage. Twenty-four hours after training, animals were put back in the chamber for contextual testing for five minutes starting immediately after the animal had been placed back into the chamber. Half of the juveniles were tested in a new chamber as a control to determine if increased freezing during testing was a result of associative learning of the cat odor to the context.

Experiment 2

Procedures for Experiment 2 were the same as Experiment 1 except that either a propranolol solution (20 mg/kg) or 0.9% saline was i.p. injected into the animal 30 minutes before exposure or immediately following exposure. This dose of propranolol is effective in young animals (Sullivan et al., 2000c; Harley et al., 2006) and pilot work done in our lab found that this was an effective dose at these ages in our experimental paradigm. When the nadolol solution (10 mg/kg) was used, it was injected immediately following exposure. 30-gauge needles were used for both infant and juvenile animals.

Experiment 3

Tissue Collection and Homogenization

For Experiment 3, only male rats were used to control for potential sex differences in CREB phosphorylation (Auger et al., 2001). Subjects were sacrificed by decapitation 60 minutes after odor exposure since peak CREB phosphorylation occurs 10-360 minutes after odor-shock pairing (Zhang et al., 2003). Nembutal (.1 mL, i.p.) was injected before

decapitation. DH and VH sections (Figure 12) were rapidly collected on cold plate (-16°C) and immediately frozen. All tissue samples are stored at -80° C until tissue homogenization. For homogenization, tissue was lysed in cold buffer containing 0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 2 mM NaF, 2 mM Na4P2O7, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.5mM PMSF, 100X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 100X phosphotase I and II inhibitor cocktails (Sigma-Aldrich) until a uniform solution was achieved. The homogenates were centrifuged for 15 min at 14000 rpm at 4°C, pellets were discarded, and protein concentrations of each sample were measured by Bradford Assay (BioRad, Hercules, CA).

Western Blot Analysis

Samples containing 50 μg of protein per lane were loaded on 10% SDS-PAGE gels and transferred by electroblotting to Immobilon-P (polyvinylidene difluoride) transfer membranes (Millipore, Bedford, MA). To probe for pCREB, membranes were pretreated with PBS 3% milk buffer and incubated overnight at 4°C in anti-phosphorylated CREB (1:1000, Milipore). Membranes were washed, treated with a secondary HRP-labeled goat anti-rabbit antibody (1:4000, Milipore) and HRPlinked anti-biotin (1:2000, Cell Signaling) for 90 min, and washed again. Membranes were then developed with ECL detection reagents (GE Healthcare) and exposed to ECL Hyperfilm (GE Healthcare). Membranes were then stripped in buffer containing 10% SDS, 0.5 M Tris-CL (pH 6.8), and 14.3 M β-ME and re-probed for CREB. To probe for CREB, membranes were pretreated with TBS-T (0.05%) 3% milk buffer and incubated overnight at 4°C in anti-CREB (1:1000, Cell Signaling). Membranes were washed, treated with a secondary HRP-labeled goat anti-rabbit antibody (1:4000, Milipore) for 60 min, and washed again. After

ECL detection, membranes were again blocked and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH – a housekeeping gene. To probe for GAPDH, membranes were pretreated with TBS-T (0.1%) 5% milk buffer and incubated overnight at 4°C in anti-GAPDH (1:2000, Cell Signaling). Membranes were washed, treated with a secondary HRP-labeled goat anti-rabbit antibody (1:4000, Milipore) for 60 min, and washed again. Membranes were then developed and exposed as previously described. Membranes were stripped between probing since the molecular weights of pCREB (43 kDa), CREB (43 kDa), and GAPDH (37 kDa) are within too narrow of a range to be run simultaneously.

2.6 Data Analysis

When a significant main effect was found without a significant interaction in two-way factorial analyses of variance (ANOVA), the Tukey HSD procedure was used to control for Type 1 error across the pairwise comparisons. When a significant interaction was found, follow-up tests were conducted to evaluate the two pairwise differences among the means with α set at .0125 (.025/2 = .0125) to control for Type I error over the two pairwise comparisons. For western blot films, quantitative densitometric analysis was performed using ImageJ software. pCREB protein levels were normalized by first dividing values for both pCREB and CREB by GAPDH, and then by dividing this new value of pCREB by the new value for CREB so that [(pCREB/GAPDH)/(CREB/GAPDH)]. Further, these values were divided by the average density of the control condition. All statistical tests were performed using SPSS (v. 16 for

Windows, Chicago, IL) and all graphs were generated using GraphPad Prism (v. 5 for Windows, San Diego, CA).

3. Results

3.1 Experiment 1: The unlearned fear response and contextual fear learning to natural predator odor in early ontogeny

The aim of this experiment was to determine if predator odor supports contextual fear learning in early life. The unlearned response of infant and juvenile rats to cat odor has been described (Wiedenmayer et al., 2005) but in the present experiment, a different odor was used that more closely mimics the actual presence of a cat.

Infant (PN14) and juvenile rats (PN26) were preexposed to the context the day before training, exposed to either cat odor (material that had been used as a cat bed) or the control odor (lavender oil) during training, and returned to the chamber for contextual testing 24 hours after training. Conditioned freezing in young rats is enhanced when animals are preexposed to the context (Pugh and Rudy, 1996). A one-trial learning paradigm was used because, from an ethological standpoint, even a single experience with a potent predator stimulus would represent enough of a threat to promote rapid, long-lasting changes in behavior (Wiedenmayer, 2004). We predicted that both infant and juvenile rats have the innate ability to recognize and respond to cat odor but that only juvenile rats form contextual fear memories.

Our data indicate that cat odor is an effective stimulus to produce unlearned, innate fear across early ontogeny. Both infants and juveniles exposed to cat odor froze significantly more than infants and juveniles exposed to the control odor (significant

main effect for stimulus: $F_{(1, 20)} = 28.42$, $p < .001$; observed power = 1.00) (Figure 13). There was no significant main effect for age ($F_{(1, 20)} = 0.02$, $p > .05$; observed power = .05) as well as no interaction between the stimulus and age ($F_{(1, 20)} = 0.00$, $p > .05$; observed power .05) (Figure 13) on freezing. Although previous findings published by our lab suggested that young animals respond to odors based on the ecological relevance of the threat to their age-group (Wiedenmayer and Barr, 2001), it was proposed that the stimulus used in the present study represents an immediate threat, severe enough to be relevant at any age. That is, that it more closely signals the presence of a real cat compared to scatological cues.

Upon return to the context the following day, juveniles previously exposed to the cat odor froze significantly more than any other group (significant 2 X 2 interaction: $F_{(1, 20)} = 6.51$, $p < .05$; observed power = .68) (Figure 14). Juvenile animals that had been exposed to cat odor froze significantly more than juveniles that had been exposed to the control odor ($p < .001$) and more than infants that had been exposed to cat odor ($p < .001$) (Figure 14). There were no significant differences in freezing in infant animals previously exposed to either cat or the control odor when they were returned to the context the following day nor were there differences between the ages when they had previously been exposed to the control odor. These results support our predications and indicate that juvenile, but not infant, rats were able to form contextual fear memories in response to the cat odor.

An alternative explanation of these data is that increased freezing on the test day does not indicate learned fear of the context but is a generalized fear response resulting from previous exposure to a severe threat. For example, rats shocked in one context and

tested in another context display levels of freezing higher than nonshocked controls (Bolles and Collier, 1976). To rule out the possibility of between-context generalized fear (as discussed by (Fanselow, 1980) and as a control to test for associative contextual learning, all experimental features of the contextual fear conditioning paradigm with one exception were retained. 24 hours later, juvenile rats were tested in a new context that differed from the training chamber in color, size and floor substrate. Juveniles previously exposed to the cat odor and tested in the same context froze significantly more than all other tested juveniles (significant 2 X 2 interaction: $F_{(1, 20)} = 6.50$, $p < .05$; observed power = .68). Juvenile animals that had been exposed to cat odor froze significantly more than juveniles that had been exposed to the control odor ($p < .001$) and, importantly, more than juveniles that had been exposed to cat odor and tested in a new context ($p < .001$) (Figure 15). There were no significant differences in freezing in juvenile animals previously exposed to either cat or the control odor when they were placed in a new context the following day nor were there differences between the two contexts when they had previously been exposed to the control odor. Thus, observed freezing in juveniles was the result of the contextual stimuli not an artifact of generalized fear. The same mechanisms that operate in the adult animal appear to be also functional in juveniles but likely not in infants that do not demonstrate contextual fear learning. The role of NE in this ability at this age was addressed in the next experiment.

3.2 Experiment 2: Effects of systemic injection of propranolol on contextual fear memory acquisition and consolidation

In Experiment 1, predator odor was found to be an effective stimulus to induce unlearned fear and contextual fear learning in juvenile rats. The first aim of Experiment 2 was to investigate the role of NE in fear memory acquisition in response to predator odor at this age.

The β-adrenergic antagonist propranolol or the vehicle was injected systemically in juveniles before exposure to the odor. We used 20 mg/kg of propranolol 30 minutes prior to exposure since this dose and injection schedule have been found to be effective in previous studies (Sullivan et al., 1989; Sullivan et al., 2000c; Weber et al., 2003). 24 hours after training, animals were returned to the chamber and tested for contextual fear. Based on Do Monte (2008), who found that propranolol blocks the unconditioned fear response in adults rats, it was predicted that juvenile rats injected with the β-adrenergic antagonist before exposure to the cat odor would freeze less during training than rats injected with the vehicle. Further, it was predicted that blocking the unconditioned fear response would prevent memory acquisition.

The predictions were confirmed as juveniles injected with propranolol before exposure to cat odor froze significantly less than vehicle-injected animals during training (significant 2 X 2 interaction: $F_{(1, 20)} = 9.81$, $p < .01$; observed power = .85; post-hoc $p < .001$) (Figure 16) and testing (significant 2 X 2 interaction: $F_{(1, 20)} = 6.82$, $p < .05$; observed power = .70; post-hoc $p < .001$) (Figure 17). There were no significant differences in freezing between animals injected with propranolol before exposure to either the cat or the control odor during training and testing. Animals injected with the

vehicle before exposure to cat odor froze significantly more than animals injected with the vehicle before exposure to the control odor during training ($p < .001$) (Figure 16) and testing ($p < .01$) (Figure 17). There were no significant differences in freezing in animals injected with either propranolol or the vehicle before exposure to the control odor during training and testing.

These results demonstrated that propranolol is effective in blocking the unlearned fear response and conditioned contextual fear memory to cat odor in juveniles. Because propranolol was administered before odor exposure and reduced the expression of fear both during training and testing, we propose that the acquisition of the fear memory was disrupted. One alternative explanation for these results could be that propranolol did not block fear but rather the ability of the animal to perceive or identify the odor on the level of sensory input as being potentially harmful. This is doubtful, however, as blockade of β-adrenergic receptors in the olfactory bulb does not impair odor discrimination, even in the young rat pup (Doucette et al., 2007). A second explanation is that propranolol affects motor output, causing animals to be hyperactive. This explanation is also unlikely, as propranolol decreases heart muscle contraction and is clinically used to reduce hypertension and performance anxiety (Brantigan et al., 1982; Sica et al., 2004). Instead, propranolol likely affects central neural processing. During acquisition, globally blocking receptors may prevent the neuronal firing necessary to attend to the threat or to provoke an adaptive emotional state (i.e. fear) by blocking β-adrenergic receptors in the amygdala. In the classic shock paradigm, the unlearned fear response cannot be measured since the animal exhibits a startle response and does not freeze until after training (Takahashi et al.,

2008). Interestingly, in the predator paradigm, freezing behavior during training indicates acquisition as blocking the unlearned fear response (freezing) also blocked conditioning.

The results of the current experiment demonstrate that NE antagonism reduced unlearned fear and consequently prevents contextual fear memory acquisition. In the next experiment, the role of NE in the consolidation of contextual fear memory was investigated. Unlearned fear of predator odor has to remain intact to allow fear memories to be formed. Therefore, the NE antagonist has to be administered after cat odor exposure in order to disrupt fear memory consolidation. Indeed, propranolol injected after training blocked the formation of contextual fear memory to cat odor (Adamec et al., 2007; Do Monte et al., 2008). Likewise, injection of propranolol immediately after training is effective in blocking shock-induced contextual learning in adult rats and olfactory associative learning in rat pups (Wilson et al., 1994; Ji et al., 2003). The second aim of Experiment 2 focused on the role of NE in fear memory consolidation in juvenile rats after exposure to predator odor.

To this end, propranolol was injected immediately after exposure. There exists a period for effective propranolol administration, soon after training, when β-adrenergic activity is at its highest (Ji et al., 2003). Wilson (1994) found that i.p. injections of propranolol were effective to block odor preference in pups when administered immediately and 60 minutes, but not at 4 hours, after training.

It was predicted that juvenile rats injected with propranolol after exposure to the cat odor would freeze less than rats injected with the vehicle to the context the following day. Animals exposed to the cat odor and injected with the vehicle were the only group to exhibit contextual learning (significant 2 X 2 interaction: $F_{(1, 20)} = 28.88$, $p < .001$;

observed power = 1.00) (Figure 18). Animals injected with propranolol after exposure to cat odor froze significantly less than animals injected with the vehicle after exposure to cat odor ($p < .001$) (Figure 18). There were no significant differences in freezing in animals injected with propranolol after exposure to either the cat or the control odor. Animals injected with the vehicle after exposure to cat odor froze significantly more than animals injected with the vehicle after exposure to the control odor ($p < .001$) (Figure 18). There were no significant differences in freezing between animals injected with either propranolol or the vehicle after exposure to the control odor.

These results indicated that propranolol effectively blocked the consolidation of long-term fear memory. Since animals were injected systemically and not locally, it is possible that this outcome was the result of propranolol blocking peripheral, not central NE receptors. In order to rule out peripheral effects of NE on the learned response, juvenile rats were systemically injected with the peripheral β-adrenergic receptor antagonist nadolol, which does not cross the blood-brain barrier (Colussi-Mas et al., 2005; Do Monte et al., 2008). The testing schedule remained the same but only cat odor was used since differences between drug, not between stimuli, were of interest. Each animal was injected with nadolol (10 mg/kg) after odor exposure since this dose was effective in studies with adult rats (Colussi-Mas et al., 2005; Do Monte et al., 2008). An independent-samples *t* test did not find significant differences in freezing between animals that had been previously exposed to cat odor and were injected with nadolol ($M = 38.52$; $SEM = 4.21$) or the vehicle ($M = 37.98$; $SEM = 8.26$) when they were returned to the context the following day, $t_{(10)} = -0.06$, $p > .05$ (Figure 19).

Overall, these results demonstrated that propranolol injected after exposure to cat odor prevented contextual fear memory formation in juveniles by blocking central, and not peripheral, β-adrenergic receptors. Alternatively, it may be that propranolol had a negative effect on short-term memory (STM) and, subsequently, the transference of the information to LTM. This scenario is unlikely, however, as Ji (2003) demonstrated that there were no differences in freezing in adult rats that were either not injected, injected with the vehicle, or injected with propranolol immediately after training when they were returned to the context 1.5 hours later. Thus, β-adrenergic blocking did not affect STM, but it did affect LTM as there were significant decreases in freezing in the propranolol-injected animals when they were re-tested after 24 hours (Ji et al., 2003). The last experiment in this series was designed to test the effects of propranolol on the hippocampal involvement in LTM by measuring a protein that mediates changes associated with LTM, pCREB.

3.3 Experiment 3: Effects of predator odor cues and propranolol on hippocampal pCREB

In Experiment 2, it was found that central NE was required for contextual fear memory acquisition and consolidation in juvenile rats. In Experiment 3, the role of hippocampal CREB, a transcription factor involved in the formation of long-term fear memories in long-term contextual fear learning and the effects of NE manipulation on its expression were examined.

Since the hippocampus is necessary for contextual learning, and juveniles, but not infants, demonstrate contextual fear learning (Experiment 1), the first aim of Experiment

3 was to investigate differences in hippocampal functioning between infants and juveniles. Experiment 1 showed that infants respond with unlearned fear to cat odor. Thus, failure to achieve contextual fear conditioning must occur during encoding, consolidation, or retrieval. CREB activity has been demonstrated in early ontogeny, with levels of pCREB in the hippocampus changing in a cell-specific way during the first weeks of life (Bender et al., 2001). It was proposed that lack of pCREB in the infant hippocampus and an increase of pCREB in the juvenile hippocampus following contextual training would indicate that it is activated as part of long-term fear memory consolidation.

Differences in pCREB levels in the hippocampi of infant and juvenile rats exposed to either cat odor or the control odor were examined. We measured CREB, pCREB, and GAPDH in DH and VH measured by western blot. Although β-adrenergic receptors are found in CA1 and CA3 and both of these regions are present in the DH and VH, they were processed separately since the region of the hippocampus that is involved in contextual conditioning remains under investigation.

It was predicted that there would be no differences in normalized pCREB levels in the DH and VH of infants exposed to the cat odor or the control odor but increased pCREB levels in the DH and VH of juveniles exposed to the cat odor since this age is able to achieve contextual conditioning. A 2 X 2 interaction (DH: $F_{(1, 20)} = 9.70$, $p < .01$; observed power = .84; VH: $F_{(1, 20)} = 5.92$, $p < .05$; observed power = .64) revealed that juveniles exhibited significantly higher pCREB levels in both the DH ($p < .001$) and VH ($p < .01$) after they were exposed to cat odor than after exposure to the control odor and compared to infants exposed to cat odor (DH: $p < .001$; VH: $p < .01$) (Figure 20). There

were no significant differences in pCREB levels in infants after exposure to either the cat or the control odor or between juveniles and infants exposed to the control odor.

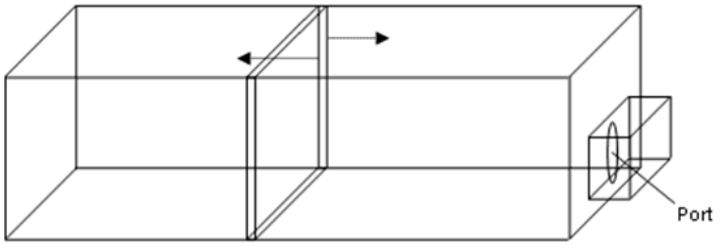
These results paralleled the behavioral findings in Experiment 1, as juvenile, but not infant rats, displayed contextual fear conditioning. Therefore CREB phosphorylation in the hippocampi of juvenile rats exposed to the cat odor could be required for the consolidation of contextual fear memory. The role of NE in this memory consolidation was explored next by systemically injecting either the vehicle or propranolol into juvenile rats immediately after exposure to either the cat odor or control odor. Experiment 2 demonstrated that propranolol blocks the consolidation of contextual fear learning. This experiment examined if the blocking of central β-adrenergic receptors also blocked CREB phosphorylation in the juvenile hippocampus. Because NE mediates contextual fear learning in juveniles, and hippocampal pCREB is involved in the consolidation of this learning, this experiment was intended to test the effects of central NE release on pCREB levels in the hippocampus. Again CREB, pCREB, and GAPDH in the DH and VH were measured by using western blot techniques.

Increased pCREB levels in the hippocampi of juvenile rats injected with the vehicle and exposed to the cat odor compared to all other groups were anticipated. There was a 2 X 2 interaction (DH: $F_{(1, 20)} = 12.92$, $p < .01$; observed power = .93; VH: $F_{(1, 20)} = 6.98$, $p < .05$; observed power = .71), in that vehicle-injected animals exhibited significantly increased pCREB levels in both the DH and VH after they were exposed to cat odor compared to cat odor-exposed propranolol-injected animals (DH: $p < .001$; VH: $p < .001$) or when they were exposed to the control odor (DH: $p < .001$; VH: $p < .01$) (Figure 21). There were no significant differences in pCREB levels in propranolol-

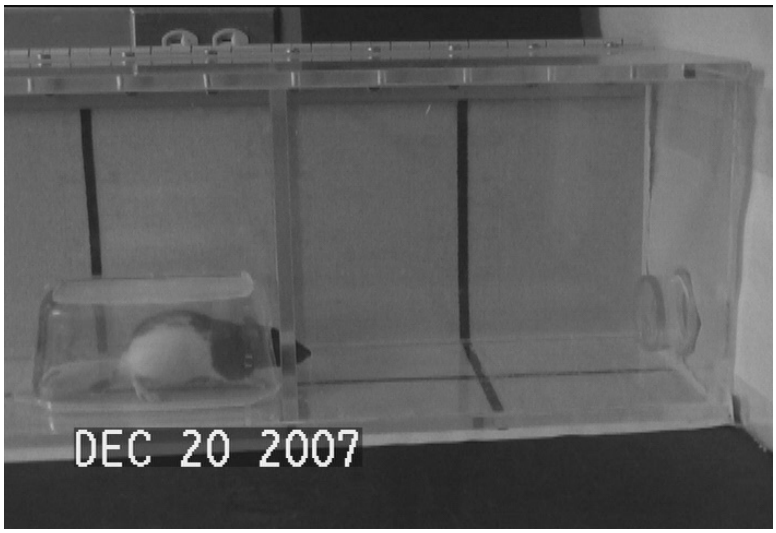
injected animals after exposure to either the cat or the control odor. There were no significant differences in pCREB levels in vehicle- and propranolol-injected animals following exposure to the control odor.

These results indicate that contextual fear memory formation is associated with increased levels of CREB activation in the hippocampus and that the NE antagonist inhibits this activation. NE participates in the activation of the transcription factor CREB in the hippocampus, which is necessary for long-term contextual fear memory formation.

A.



B.



C.

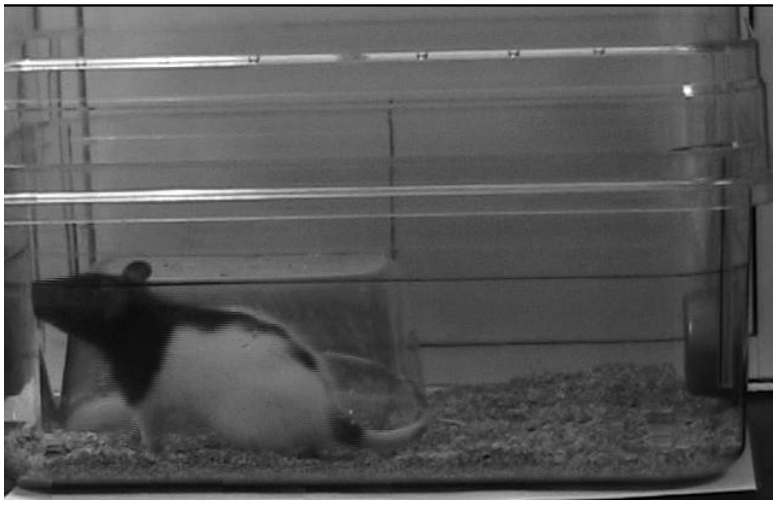


Figure 11. Odor conditioning apparatus with hidebox. Rats are exposed to cat cues that are presented at the odor port (A). The size of the chamber is adjusted by a divider wall. A hide box can be accessed through an opening in the divider wall (B). The different testing chamber to control for generalized anxiety (C).

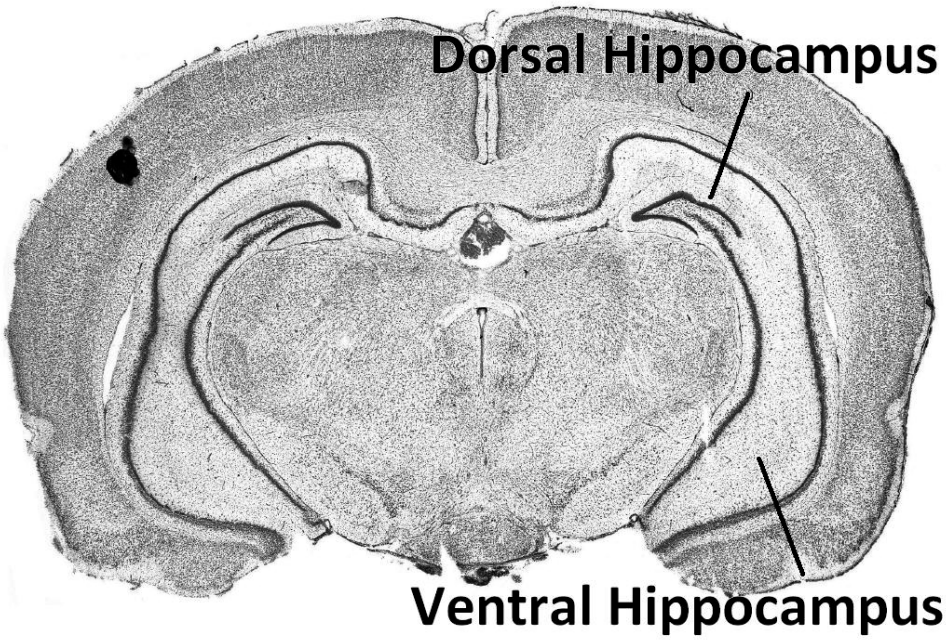


Figure 12. Dorsal and ventral hippocampus location. Sites of tissue collection for western blot analysis.

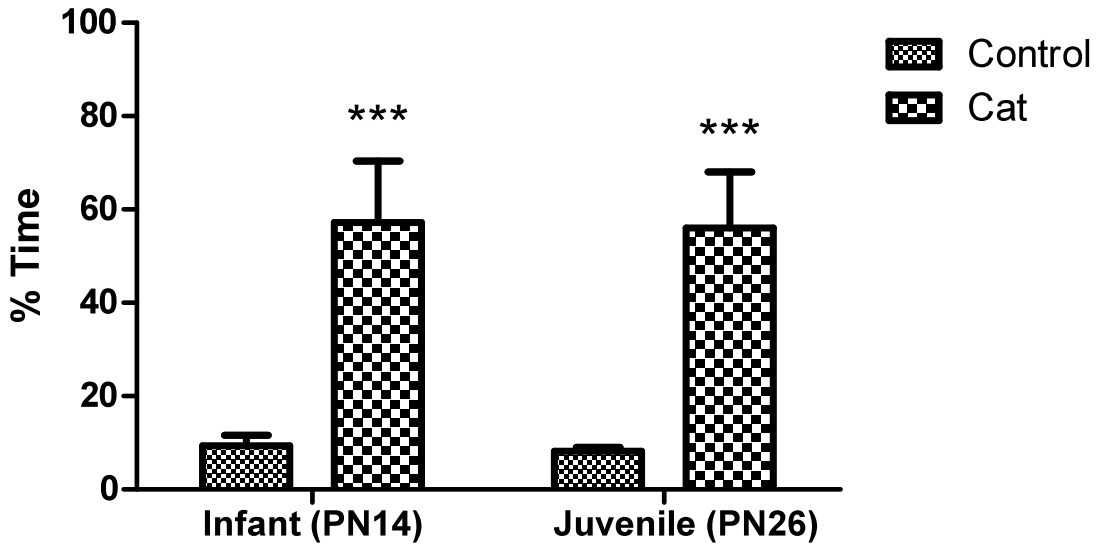


Figure 13. Cat odor is a highly effective fear-inducing stimulus across early ontogeny. Mean and SEM percent time freezing during odor exposure in Experiment 1 (*** $p < .001$, cat odor significantly different from the control odor; $N = 6$ per group).

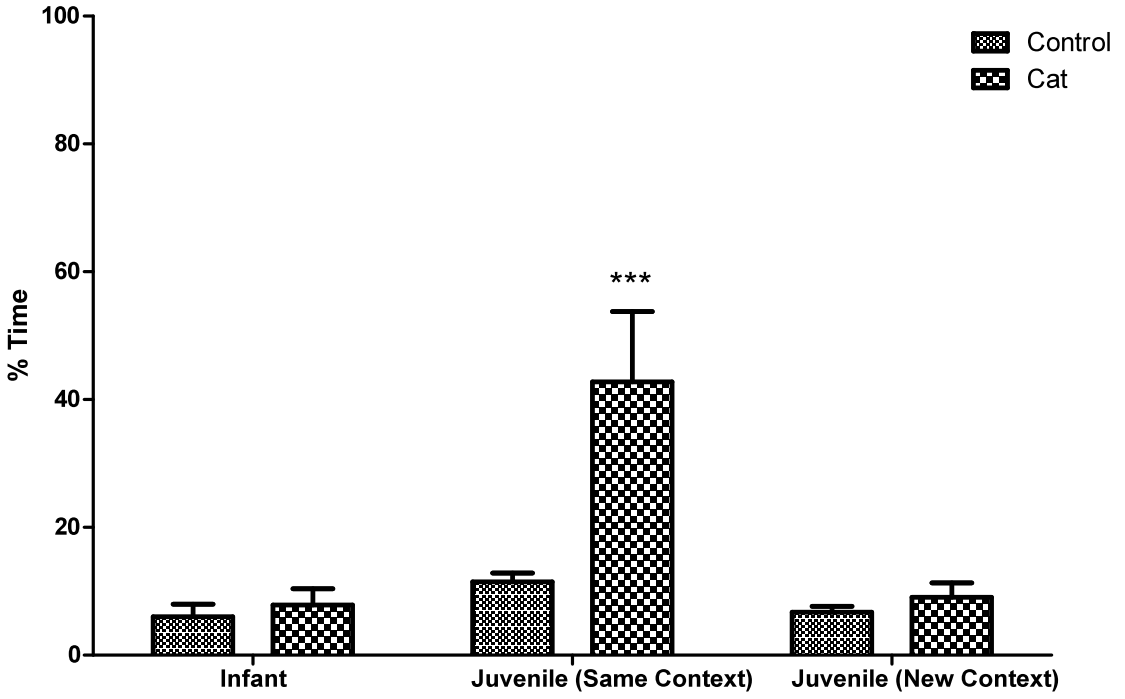


Figure 14. Juvenile, but not infant, rats were able to exhibit fear memory when returned to the context 24 hours after exposure to the cat odor. When juveniles were tested in a new context 24 hours after exposure to the cat odor, they did not display an increase in freezing. Mean and SEM percent time freezing during contextual test in Experiment 1 (*** $p < .001$, significantly different from every other group; $N = 6$ per group).

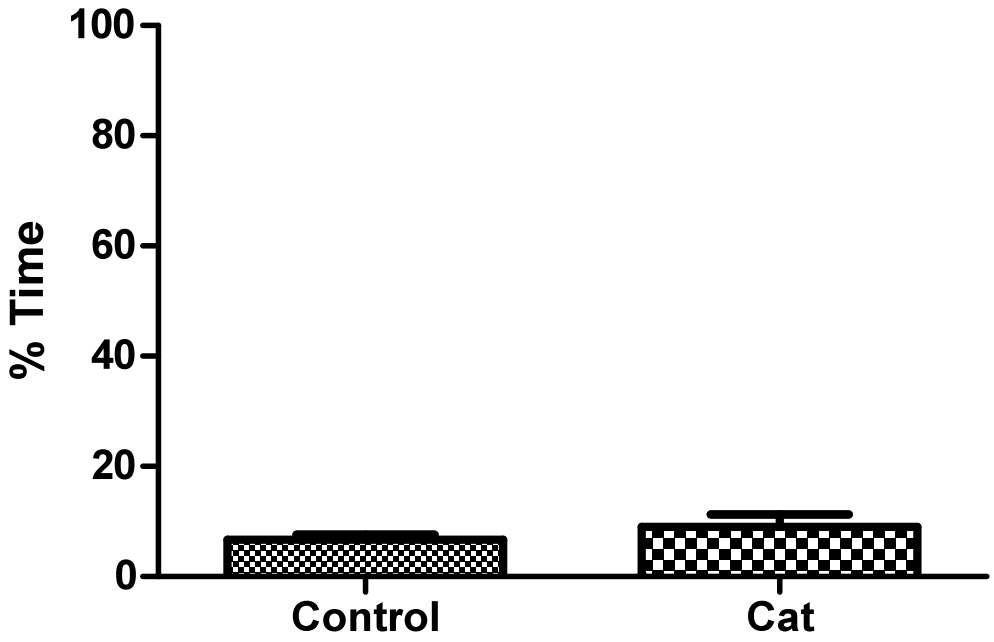


Figure 15. Juvenile rats did not form fear memories when placed in a novel context 24 hours after exposure to the cat odor. Mean and SEM percent time freezing during contextual test in Experiment 1 (N = 6 per group).

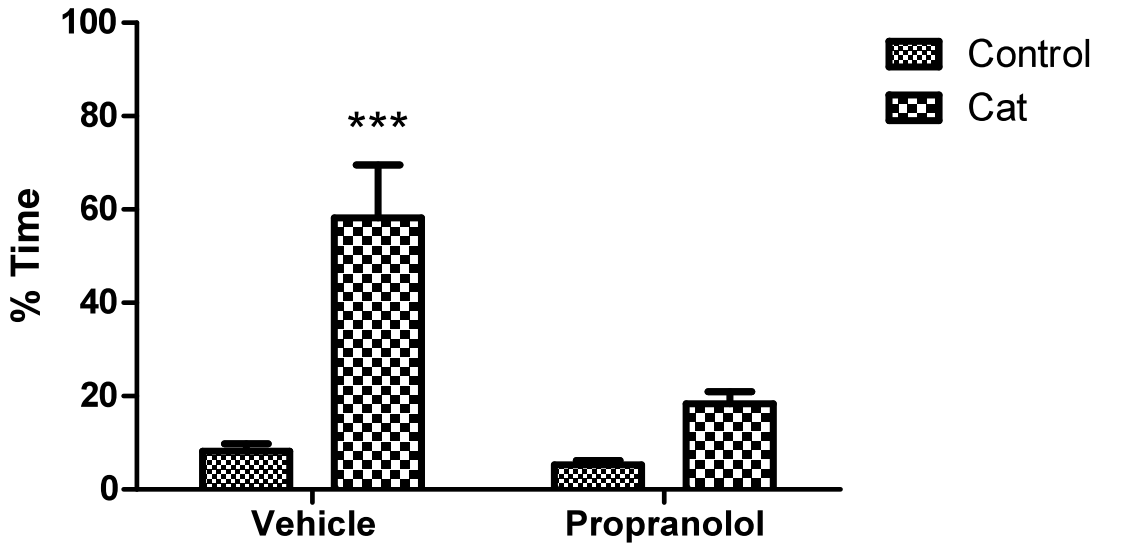


Figure 16. The β-adrenergic antagonist propranolol blocks juveniles' unlearned fear response to cat odor when it is administered before exposure. Mean and SEM percent time freezing during odor exposure in Experiment 2. Rats were injected with 20 mg/kg of propranolol or the vehicle 30 min prior to exposure (*** $p < .001$, significantly different from every other group; $N = 6$ per group).

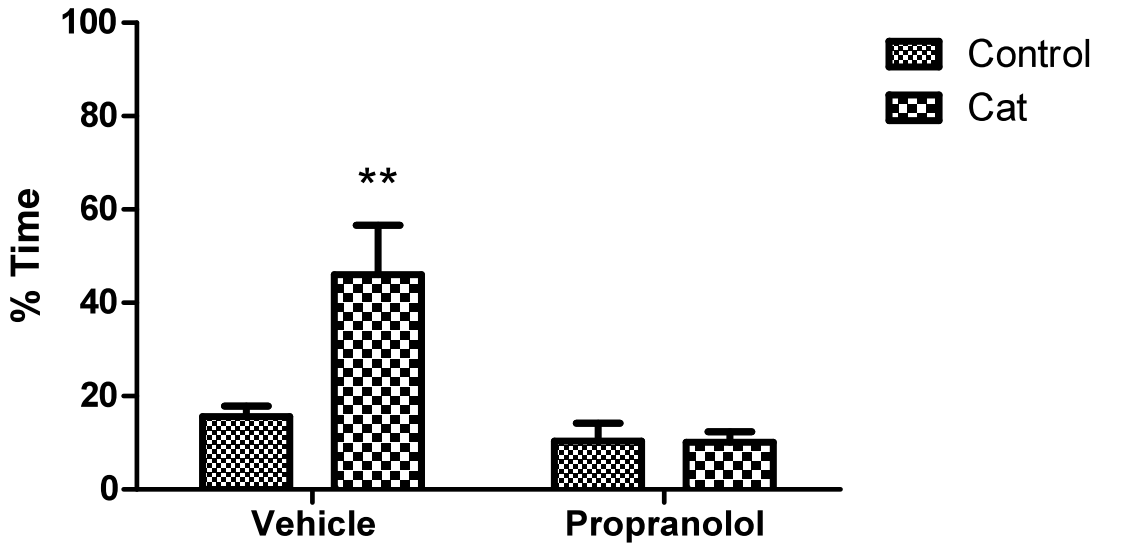


Figure 17. Pretraining injection of the β-adrenergic antagonist propranolol blocks contextual fear learning to cat odor. Mean and SEM percent time freezing during testing in Experiment 2. Rats were injected with 20 mg/kg of propranolol or the vehicle 30 min prior to exposure (*** $p < .01$, significantly different from every other group; $N =$ 6 per group).

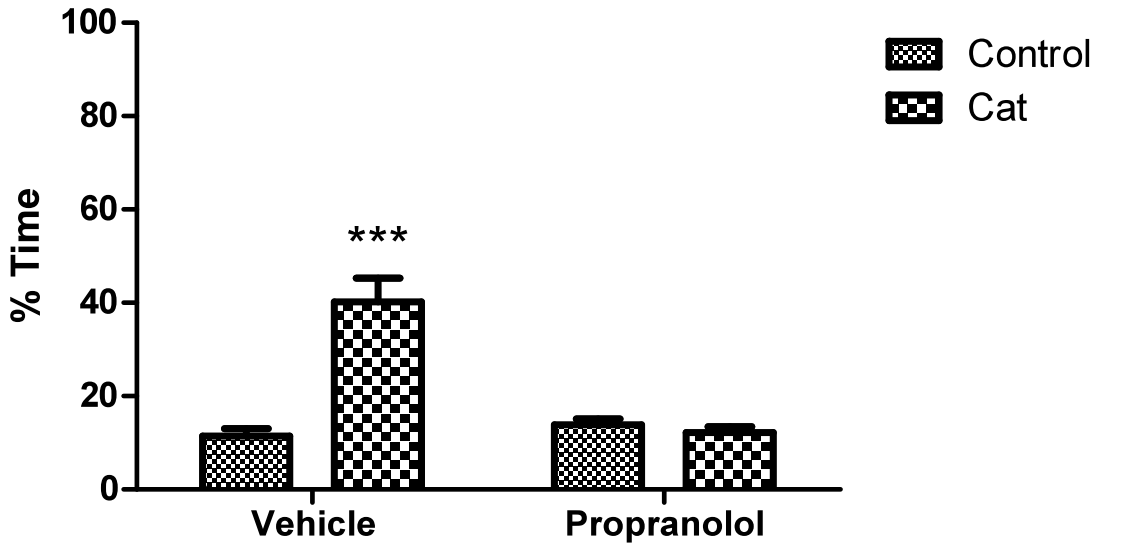


Figure 18. The β-adrenergic antagonist propranolol effectively blocks the consolidation of long-term fear memory in juveniles. Mean and SEM percent time freezing during contextual test in Experiment 2. Rats were injected with 20 mg/kg of propranolol or vehicle immediately following training and tested 24 h later (*** $p < .001$, significantly different from every other group; $N = 6$ per group).

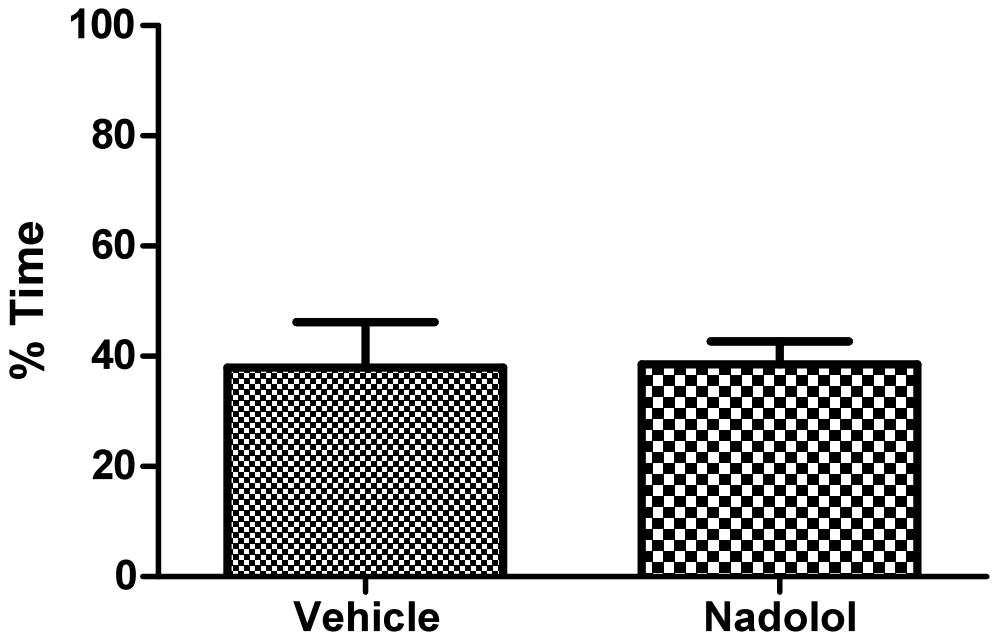


Figure 19. The peripheral β-antagonist nadolol has no effect on contextual fear learning in juveniles exposed to cat odor. Mean and SEM percent time freezing during contextual test in Experiment 2. Rats were injected with 10 mg/kg of nadolol or vehicle immediately following training and tested 24 h later ($N = 6$ per group).

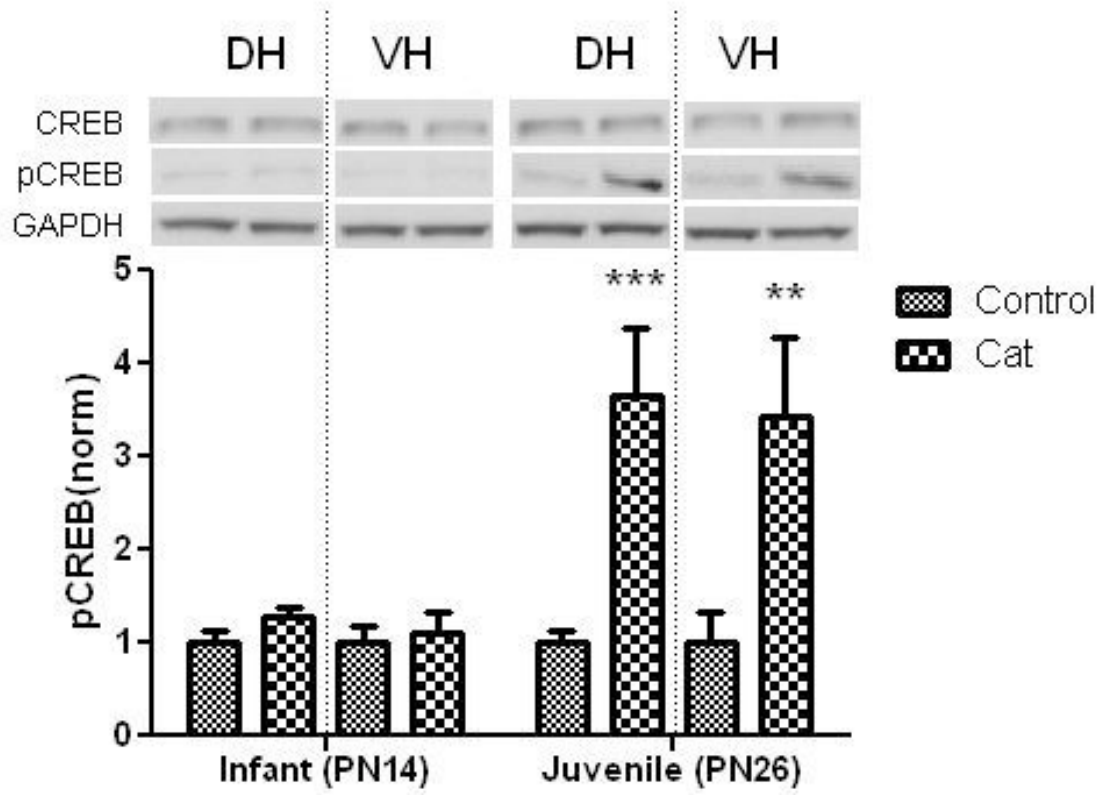


Figure 20. Juveniles exhibited a significant increase in CREB phosphorylation in both the dorsal (DH) and ventral hippocampus (VH) after they were exposed to cat odor than when they were exposed to the control odor. Mean and SEM normalized pCREB levels in DH and VH in Experiment 3 (** $p < .01$, *** $p < .001$, significantly different from all other groups in that brain area; $N = 6$ per group). A representative western blot film is shown for each age and condition above the corresponding bar.

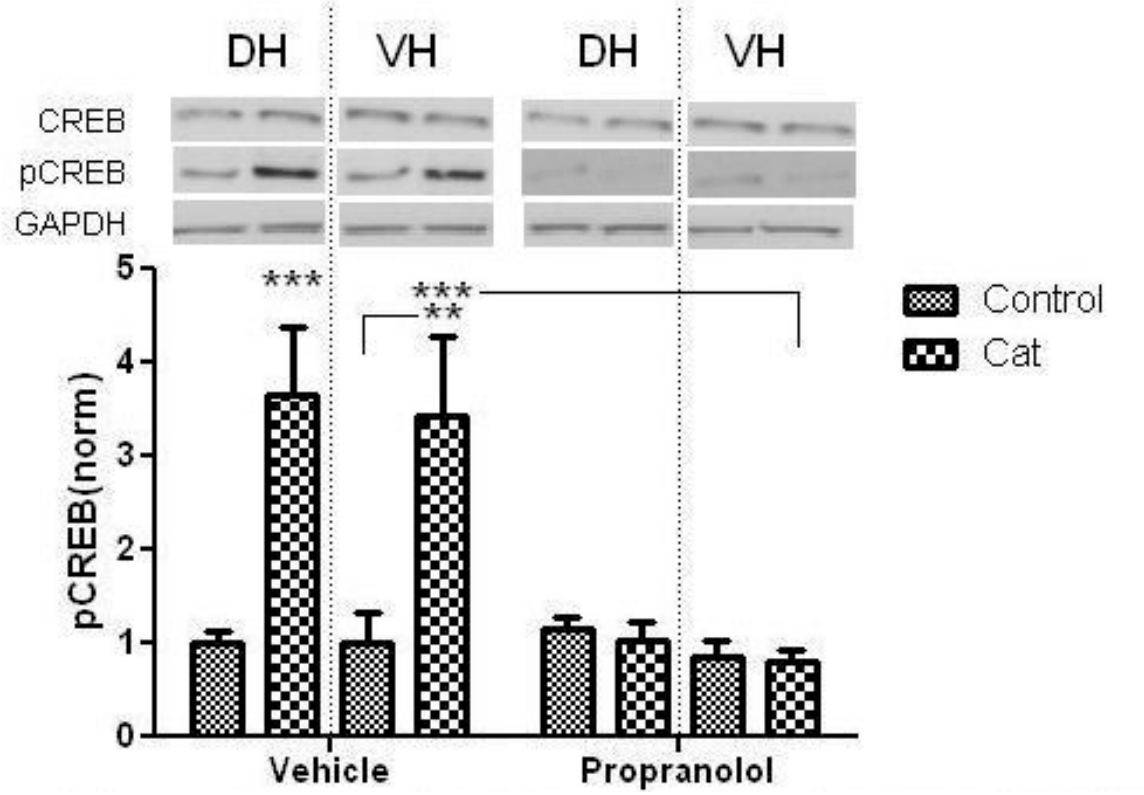


Figure 21. Propranolol blocked the increase in CREB phosphorylation in both the DH and VH of juveniles after they were exposed to cat odor. Mean and SEM normalized pCREB levels in DH and VH in Experiment 3. Rats were injected with 20 mg/kg of propranolol or the vehicle immediately following training (** $p < .01$, *** $p < .001$, significant differences in pCREB in cat odor-exposed, vehicle-injected animals from all other groups in the DH and from cat odor-exposed, propranolol-injected and control odor-exposed, vehicle-injected animals in the VH; N = 6 per group). A representative western blot film is shown for each age and condition above the corresponding bar.

4. Discussion

These experiments examined the role of the noradrenergic system and CREB in hippocampal-dependent contextual fear learning during early ontogeny. Results can be concisely summarized as follows. In the first experiment, natural predator odor cues were effective in eliciting an unlearned fear response in both infant and juvenile rats, however contextual fear learning was only observed in juveniles. The second experiment showed that systemic injection of the β-adrenergic antagonist propranolol was effective in reducing the unlearned fear response and in preventing fear memory acquisition and consolidation in juveniles. Also, pCREB, a necessary transcription factor for LTM formation, was elevated in the hippocampi of juveniles, but not of infants, exposed to the cat odor in the third experiment. This increase was blocked by systemic injection of propranolol, indicating that NE is required for CREB phosphorylation. Taken together, these results suggest that centrally-released NE facilitates contextual fear conditioning by inducing CREB phosphorylation in juvenile rats and that absence of this effect may contribute to failure of contextual fear learning in infant rats.

Chapter 4: Conclusion

Experiments presented in this thesis were designed to develop a contextual fear-learning paradigm utilizing ecologically relevant stimuli that can be used for young rats. We then used this paradigm to test the hypotheses that the noradrenergic system is involved in contextual fear learning in juvenile, but not infant, rats and that this contextual learning is hippocampal-dependent. Cat odor can be used to induce both unlearned and contextually conditioned fear in adults. It was also known that contextual learning requires a mature, functioning hippocampus. Not known was how and when the hippocampus is activated during early life and during contextual fear learning. Focusing on early life, our results indicate that cat odor produces unlearned, innate fear in infant and juvenile rats, but contextual fear learning only in juveniles. Learning in juveniles was coincident with increased CREB phosphorylation and both effects were blocked with β-adrenergic antagonist propranolol administration. Taken together, these results indicate that, although innate fear occurs within the neonatal period, contextual fear learning is a relatively late-occurring event, is hippocampal dependent, and mediated by NE. These findings compliment and extend the established animal models of contextual fear learning in adults.

The first study used to address Aim 1 provides evidence that unlearned fear behavior in rats changed as a function of age and the stimulus, though not entirely as expected. Infant and juvenile rats displayed fear-related behavior to cat odors by freezing for more time than adolescents and adults. Contrary to our expectations, adolescents and adults did not significantly use risk-assessment as a defensive strategy more than infants and juveniles. One possible explanation for the lack of differences in risk-assessment

behaviors in the older animals is that, in response to cat odor, the adult rat's natural defensive strategy is to hide and then engage in freezing and vigilant risk-assessment in order to decrease conspicuousness and attend to the threat (Blanchard and Blanchard, 1989; Dielenberg and McGregor, 2001). Without a place to hide, the rat may engage in a variety of other behaviors such as exploration as they seek out a hiding place. Sigmundi (1997) and de Oca et al. (2007) have gone so far to suggest that flight to an enclosure and freezing are both part of a "single defensive strategy". Indeed, the levels of freezing to cat fur in adults were lower than have been reported elsewhere (Li et al., 2004; Takahashi et al., 2007). The sum of the behaviors that make up the single strategy of risk-assessment may also have introduced variability, making it a less reliable a measure of unlearned fear-related behavior than freezing. For these reasons, a hide box was added to later experiments.

The cat fur odor used in the first study proved to be a potent fear-producing stimulus in infants and juveniles. Because this stimulus was not purely cat fur but also likely contained other cat odors, this should be regarded as a mixed stimulus. Although conclusions can be drawn between cat urine and the control odor or the cat odor mix and the control odor, differences in the fear response between cat urine and the cat odor mix should be interpreted with caution. That is, it likely was not that young animals were better able to differentiate between the two cat stimuli than adolescents and adults, especially since the older animals displayed admittedly low levels of freezing to both cat odors. Contrary to our expectations, the size of the chamber where animals were exposed had no effect on fear-related behavior. Future work in this area will be discussed below.

The second study used to address Aim 2 demonstrates that, in addition to producing unlearned fear in both infant and juvenile rats, cat odor supports contextual fear learning in juveniles. One advantage of the predator odor exposure model is that the same behavior can be measured and compared during training and testing. Predator odors additionally facilitate the interpretation of the development of unlearned and learned fear. For instance, the juvenile rats' ability to form long-lasting contextual fear memories is likely the consequence of natural selection. In their life history, young rats become more mobile after weaning and begin to venture from the natal burrow (Barnett, 1958; Bolles and Woods, 1964). Contextual learning ability may be critical to their survival since the location where a threat was encountered is a reliable predictor for future threat. In contrast, the infant rat is typically confined to the nest with minimal locomotor activity (Nowak, 1999). Therefore, the infant is under little selection pressure to learn about the context. Consequently, brain circuits may not be mature at this age.

Aim 3, also addressed by the second study, examined the development of these brain circuits. Results showed that the β-adrenergic antagonist propranolol reduces the unlearned fear response and prevents fear memory acquisition and consolidation in juveniles. NE thus appears to be critical for learning throughout life, even as the neural substrates that support different types of learning change. In the newborn, the olfactory bulb mediates cued conditioning and Sullivan (2000c) found that NE activation mediates the storage of early odor memories in this structure. Around PN10, cued associative learning is no longer supported by the olfactory bulb as autoexcitation decreases and autoinhibition emerges in the LC (Moriceau and Sullivan, 2004). Instead, the amygdala begins to mediate associative learning as major nuclei subdivisions mature (Sullivan,

2001). The amygdala is necessary for cued olfactory fear learning (Otto et al., 2000) whereas contextual fear conditioning additionally requires activation of the hippocampus (Phillips and LeDoux, 1992). Although rats can be contextually conditioned to shock when the DH is lesioned using elemental cues, several training trials are usually required for this type of learning to take place (Maren et al., 1997; Rudy et al., 2004). Since our experimental paradigm consisted of a single pairing, it is likely that contextual or configural learning took place, mediated by the hippocampus.

Since both the hippocampus and amygdala are densely populated with β-adrenergic receptors (Do Monte et al., 2008; Qu et al., 2008), NE may promote contextual learning by binding in either site. However, propranolol is effective in reducing fear memory consolidation associated with a context but not with a cue (Grillon et al., 2004), which suggests that NE critically affects the hippocampus in this type of learning. Although β-adrenergic antagonists injected into the amygdala block auditory fear memory consolidation, local injection into CA1 blocks contextual fear memory consolidation (Ji et al., 2003; Debiec and Ledoux, 2004; Qu et al., 2008).

If the hippocampus is indeed a site of contextual fear memory consolidation, pCREB levels should be enhanced in the hippocampi of contextually conditioned animals. Indeed, pCREB is increased in the hippocampi of rat pups trained in a water maze (Yang et al., 2009) and adults exposed to predator odor (Wang et al., 2007). Because pCREB is only expressed in the hippocampus when the spatial context is novel (Moncada and Viola, 2006), pCREB appears to participate in memory formation not retrieval. The data from the current series support this hypothesis as pCREB levels were elevated in juveniles exposed to the predator odor. Levels were not elevated in infants,

who are not able to achieve contextual conditioning. In addition, levels of pCREB decreased with systemic injection of the NE antagonist, supporting previous findings that NE has the ability to promote CREB phosphorylation (McLean and Harley, 2004). Thus, NE may mediate long-term contextual memory formation through the activation of the β-adrenergic receptors in the hippocampus. The results presented here support the hypothesis that contextual conditioning requires both the DH and VH (Otto and Poon, 2006) as pCREB activity was increased in both of these areas. Indeed, hippocampal field may be more critical than area as CA3 is involved in contextual memory acquisition (Lee and Kesner, 2004; Daumas et al., 2005) and CA1 in consolidation (Daumas et al., 2005).

The inability of infant animals to achieve contextual fear conditioning has been previously demonstrated and stems from immature "neural substrates" (Rudy and Morledge, 1994) and/or a "lack of functional connectivity between brain areas" (Sullivan, 2001). Yet little is known about the maturation of the hippocampus and how it relates to the limits of learning in the growing animal. Burman (2009) suggested that both the hippocampus and amygdala are functional at PN17. However, the connectivity between these two areas has not been fully established and is the subject of much current research (Huff et al., 2005; Fanselow and Dong, 2010). Results indicate that the intracellular cascade that is induced by NE binding and results in the phosphorylation of CREB might not yet be functional in infant rats.

Alternatively, the absence of change in hippocampal pCREB levels could be due to decreased NE release or binding or a lack of presynaptic plasticity (Dumas, 2005). However, NE binding in the hippocampus occurs as early as PN3 in rats (Teicher et al., 1986) and infant rats (PN10) express hippocampal pCREB (Bender et al., 2001).

Therefore, between NE binding and CREB phosphorylation, some other factor, such as an element in the cAMP/PKA cascade (McLean and Harley, 2004) may not be functional. This study may be a first step in discovering the lack of maturation in the intracellular signaling pathway. Although NE release in the amygdala is critical for the formation of long-term contextual fear memory (McGaugh and Roozendaal, 2002; Kogan and Richter-Levin, 2008), activation of the β-adrenergic receptors in the hippocampus could also be critical for this type of learning.

Although one aspect of the physiology of fear learning was investigated in this thesis, knowledge of underlying neural mechanisms is necessary for a full understanding of memory formation and for the design of interventions to alleviate the consequences of traumatic fear. For example, in humans, centrally released NE plays a critical role in emotional learning and its manipulation is promising for the treatment of traumatic memories (Fendt and Fanselow, 1999), including those memory systems involved in posttraumatic stress disorder (PTSD) (Bremner et al., 2007). Administration of propranolol appears to decrease the retention of emotional-laden material and, given immediately after trauma, reduced rates of PTSD (LaBar and Cabeza, 2006). In addition, propranolol has also been found to selectively reduce fear memory for context but not for cues (Pitman et al., 2002; Vaiva et al., 2003). Thus, it appears that the mechanisms of contextual fear learning are similar for adult humans and adult rats.

Because early trauma occurs often and can lead to later pathology and inappropriate coping mechanisms (Teicher et al., 2003), a critical challenge is to find mechanisms that are similar between young animals and human children. However, most fear learning paradigms overwhelmingly use adult animals and electric shock as the US

(but see (Tsoory et al., 2007a; Wright et al., 2008) even though it has been suggested that ecologically-relevant stimuli may better model human trauma (Dielenberg and McGregor, 2001; Adamec et al., 2007). Predator odors induced unconditioned fear across ontogeny and provided the opportunity to study the mechanisms underlying unlearned fear. These fear behaviors can become pathological when responsivity is exaggerated and anxiety becomes abnormal (Lima, 1998; Nesse, 1999; Ohman and Mineka, 2001). In these cases, long-term stimulation-induced changes model human psychiatric disorders (Rosen and Schulkin, 1998; LeDoux, 2000; Shekhar et al., 2001). Future studies should further address how natural stressors induce innate and learned fear across development.

It remains unknown if pCREB levels in the amygdala of young animals during contextual fear memory consolidation are elevated as they are in adults (Hubbard et al., 2007) and responsive to NE manipulation. Our preliminary data suggest that pCREB is elevated in the BLA of infants exposed to cat odor and that this increase is blocked with propranolol. It is possible, then, that changes in hippocampal pCREB are mediated not by NE binding to the hippocampus directly, but via the amygdala. Although a limitation of the current study is causality between propranolol administration and the reduction of hippocampal pCREB, other studies have shown that infusion of propranolol into CA1 interferes with contextual fear learning in adult mice and rats (Ji et al., 2003). These findings support the central hypothesis that activation of the noradrenergic system supports contextual fear learning by elevating levels of hippocampal pCREB, although future research should further investigate the role of NE in contextual odor fear learning in the hippocampus and amygdala via local injection. Additionally, elements that are

potentially missing in the molecular cascade between the binding of NE and pCREB level in infant hippocampi should be explored.

In sum, the experiments presented here demonstrated that predator odor can be used to promote contextual fear learning in early ontogeny and that the central release of NE facilitates acquisition and consolidation of this memory through hippocampal CREB activation. Evidence that infant rats' inability to form contextual fear memory is associated with a lack in hippocampal CREB phosphorylation was also provided. These findings may lead to a better understanding of the mechanisms that underlie this ability in early life and lead to appropriate pharmaceutical interventions for children who have experienced trauma.

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