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Individual difference in cue valuation, decision-making, and response to dopamine treatment

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Individual differences in cue valuation, decision-making, and response to dopamine treatment

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Dedication

To my parents for teaching me to ask questions and look for my own answers.

To my grandparents for always reminding me that they were proud.

Finally, to my husband who has been beside me on the journey these past five years.

Here's to our next adventure.

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Individual differences in cue valuation, decision-making, and response to dopamine treatment

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After multiple pairings of a cue and a rewarding event, animals will begin to attend to both the reward and the cue. Reports from Brown and Jenkins (1968) first described pigeons that began to track key lights predictive of food reward. Subsequently the phenomenon of conditioned cue approach has been reported across a variety of species including pigeons, quail, rats, monkeys, and stickleback fish (Brown and Jenkins, 1968; Cetinkaya and Domjan, 2006; Holland, 1977; Jenkins and Rowland, 1996; Sidman and Fletcher, 1968). More recently, investigations of individual differences in the expression of these behaviors have begun, as well as exploration into how these differences relate to other cognitive and neurological variations (Lesaint et al., 2014; Lovic et al., 2011; Meyer et al., 2012; Paolone et al., 2013). The objective of this dissertation was to characterize individual differences in rats' propensity for orienting towards a light-cue predictive of reward. I also aimed to describe how these differences related to the behavior's vulnerability to memory updating, extinction learning, a variety of cognitive functions, and behavioral and neurological responses to drug challenge. I report that all rats showed conditioned approach toward the site of food-reward delivery, but only a subset also showed robust rearing and/or orienting toward a light predictive of food (Orienters). Those rats that showed only conditioned reward approach were termed

Nonorienters. Following memory update procedures, Orienters were more likely than Nonorienters to attenuate conditioned food approach, though conditioned rearing remains unaffected. Orienters were also more likely to make impulsive and risky decisions, enter a novel and risky environment, and be distracted during an attention assay. They also emitted more ultrasonic vocalizations than Nonorienters when exposed to amphetamine. Moreover, while both Orienters and Nonorienters preferred a context previously paired with drug to a context paired with saline, Orienters emitted more ultrasonic vocalizations during the preference test. Finally, while Orienters and Nonorienters showed behavioral differences after amphetamine injection, these differences were not reflected in the activity of the brain regions responsible for the conditioned orienting response. Overall, these findings suggest that Orienters are more apt to memory update, make more impulsive and risky decisions, are more vulnerable to distraction, and that amphetamine has more impact upon the behavior of Orienters.

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Chapter 1: Background

Although species-typical responses to classical conditioning procedures have been incredibly well characterized over the last century of experimentation, variation in individual subjects' rate of responding and potential preferences for any specific type of conditioned response has been somewhat neglected, as has investigation of how variation in the acquisition of distinct conditioned responses might relate to variation in response to extinction or memory-updating protocols. Only recently have attempts been made to characterize these differences and relate them to broader behavioral phenotypes and neurochemical distinctions (Meyer et al., 2012; Robinson et al., 2014; Tomie et al., 1998, 2000). While it is known that appetitive cues predictive of food, drug, and sexual reinforcement exhibit remarkable control over animals' behavior, it has been suggested that these associations can be strengthened to the point of being maladaptive and that when this natural reward system is co-opted by experience with, for example, drugs of abuse, it can override frontocortical areas of reason and judgment. For this reason, it is logical to suppose that animals more strongly engaged with appetitive cues might afford those cues enhanced behavioral control and show evidence of decreased frontocortical control over behavior. Also, both the cue-approach response described here and the major reward pathways are dopamine dependent, suggesting a potential interaction between the two systems.

1.1. The orienting response

The idea of an "orienting reflex" or "orienting response" (OR) was born in the laboratory and writings of Ivan Pavlov (1927). Described as a "what is it" response, the

OR, characterized by dogs' perking of ears and directing of gaze toward any novel stimulus, was first viewed as a nuisance response, a response that distracted from the conditioned responses being trained in the laboratory. In 1963, Sokolov published his seminal work proposing a role of OR, suggesting that it serves to enhance the gathering of sensory information (Sokolov, 1963). Sokolov also suggested a few important characteristics of the OR: that it is dependent upon stimulus novelty, that repeated presentation of the stimulus would produce rapid habituation, and that if some characteristics of the stimulus changed (e.g. its intensity, timing, or location) the OR would return (Siddle et al., 1983; Sokolov, 2002, p. 19). As changes in sensory sensitivity were difficult to measure, ORs were most typically inferred from motor (e.g. ocular motion, ear pricking), electroencephalography (EEG), and autonomic nervous system changes (e.g. vasodilation, electrodermal response, and pupil dilation).

In an update of his original characterization, Sokolov (1966) suggested that the OR might also enhance memory for events occurring after the OR. Shek and Spinks (1985) showed that when tasked with remembering lists of words after exposure to a visual stimulus, human participants were better at remembering words that followed stimuli eliciting larger ORs. As OR research progressed, OR's dependence upon stimulus novelty came under fire. First, multiple reports showed that the OR does not always habituate. Secondly, a priori definitions of stimulus novelty are difficult to make and test. Finally, animals are exposed to novel stimuli every day yet do not attend to all of them (for review see Buzsaki, 1982). Based upon these observations, it was suggested that stimulus salience, not novelty, was necessary for the evocation of the OR. This shift in focus from stimulus novelty to salience strengthened the claim that enhancement in sensitivity might aid in the gathering not only of information regarding characteristics of the eliciting stimulus, but also any events that might follow that stimulus (Spinks and

Siddle, 1983). Kahneman (1973) suggested that components of the OR may be uniquely responsible for inhibiting ongoing activity or diverting attention toward signals which may serve as predictors of important future stimuli. Gale and Ax (1968) showed that OR can be conditioned after its association with a biologically relevant stimulus. These findings set the stage for investigations in the role of OR in classical conditioning.

1.2. CONDITIONED CUE APPROACH

Developing somewhat in parallel with the research on the OR was research on conditioned cue approach (also called "autoshaping"). When cues (conditioned stimuli, CS) and reinforcers (unconditioned stimuli, US) are repeatedly paired, characteristic conditioned responses emerge. Beginning in the late 1940s, various groups described animals that engaged in "superstitious conditioning," displaying conditioned responses (CR) that were not necessary for the delivery of a rewarding unconditioned stimulus, and in some cases actually delayed obtainment of the reward (Skinner, 1948; Morse and Skinner, 1957; Williams and Williams, 1969). In 1968, Brown and Jenkins described pigeons that, after multiple pairings of a key-light conditioned stimulus (CS) and grain unconditioned stimulus (US), showed conditioned CS approach. After a single session of classical Pavlovian conditioning, these pigeons showed robust CS-directed movement and pecking of the key-light during its illumination (also called "sign-tracking") (Brown and Jenkins, 1968; Hearst and Jenkins, 1974).

From the early conditioning literature, two theories regarding the genesis of cue-approach behaviors emerged. Pavlov's (1927) stimulus substitution theory posited that the CS serves as a surrogate US and that the form of the CR is determined entirely by the characteristics and modality of the US. However, this idea was refuted by reports that

CSs of different modalities produce different CRs, even when those CSs predict an identical US (Holland, 1977; Timberlake and Grant, 1975). This provides evidence that the CS is not simply acting as a proxy for the US, but is producing a more general motivated state that prepares subjects for engagement in consummatory behavior (Konorski, 1967; Timberlake and Grant, 1975).

Peter Holland's work throughout the 1970s characterized rats' conditioned orienting response to a light-CS predictive of food, showing that location and diffusion/localizability of the cue influenced the display of cue approach and orienting behavior (Holland, 1977). Later collaborative work determined the neural circuitry responsible for conditioned orienting to a light cue, showing that dopaminergic circuitry of the amygdala central nucleus, substantia nigra pars compacta, ventral tegmental area, and dorsolateral striatum contributed to the acquisition and expression of this response (El-Amamy and Holland, 2006, 2007; Gallagher et al., 1990; Han et al, 1997; Lee et al, 2005, 2011).

Perhaps unsurprisingly, animals also learn to approach the site of reward delivery during presentation of a cue. This approach is a behavioral indication that the subject has learned the cue-reward association (Berridge et al., 2009). In rats, when pairing light with food, cue approach and goal approach responses rely on separate neural circuitries. The amygdalo-nigral circuitry is not necessary for conditioned approach to the site of reinforcer delivery and this neural segregation allows for the possibility that these two behaviors may be dissociated under certain conditions (El-Amamy and Holland, 2006, 2007; Gallagher et al., 1990; Han et al, 1997; Lee et al, 2005, 2011).

Findings that cue-directed CRs are dependent upon characteristics of the CS but not US, that conditioned responses to CSs paired with a reinforcer are frequently identical to the original unconditioned responses to those stimuli, and that stimulus salience, not

stimulus novelty, was necessary for eliciting an OR, led György Buzsaki to propose that autoshaping was simply enhanced OR (Buzsaki, 1982; Buzsaki et al., 1979). Until this time, the two lines or research had not intersected. In his 1982 paper Buzsaki asserts that the autoshaped response is but a CS-directed response arising from the learned salience of that cue – a definition that could also describe the OR.

1.3. EXTINCTION AND MEMORY UPDATE

Repeated pairings of a CS and US elicit characteristic conditioned responses, but these responses can be attenuated if the CS is then presented in the absence of the US reinforcer; however, this attenuation ("extinction") of conditioned responding is not permanent, and the CR may return if the animal is returned to the conditioning context, exposed to an unsignaled US, or after the simple passage of time (phenomena termed renewal, reinstatement, and spontaneous recovery) (Pavlov, 1927; Rescorla and Heth, 1975; Bouton and Bolles, 1979; Robbins, 1990; Bouton, 2002). These findings suggest that instead of erasing or updating the original conditioning memory, extinction training forms a second, competing memory. The return of conditioned responding during tests for renewal, reinstatement, and spontaneous recovery is believed to indicate a reversion to the original CS-US pairing memory (Bouton, 2004).

While extinction fails to permanently attenuate conditioned responding, interventions aimed at preventing the reconsolidation of aversive memories have been shown to accomplish this attenuation. After a memory is formed and consolidated, it can be retrieved. Upon retrieval, the memory is then in a labile state requiring reconsolidation (McGaugh, 2000; Dudai, 2006). Pharmacological manipulations that disrupt the protein synthesis necessary for reconsolidation have successfully prevented

the return of conditioned responses, presumably by weakening the CS-US memory during its state of retrieval-induced lability (Nader et al., 2000; Nader, 2003; Tronson and Taylor, 2007). Recently, behavioral methods have been identified which also attenuate the return of conditioned fear (Monfils et al, 2009; Schiller et al., 2010). Monfils et al. (2009) describes a behavioral paradigm in which an extinction session presented after memory retrieval permanently attenuates conditioned fear responses in rats. As the memory is in a labile state following the retrieval trial, the extinction session that follows is believed to update the original CS-US memory to a CS-no US memory. The retrieval + extinction manipulation has also been used to attenuate drug-seeking behavior (Xue et al., 2012). However, this investigation into the manipulation's ability to attenuate appetitive conditioned responding did not investigate how the manipulation might differ in its impact on cue-directed vs. reinforcer-directed responding.

1.4. IMPULSIVE BEHAVIOR

It has been suggested that cue approach behaviors represent a lack of inhibitory control, exemplified by cases in which cue approach or engagement actually precludes receipt of the rewarding US (Williams and Williams, 1969; Breland and Breland, 1961). Deficits of inhibitory control are associated with impulsive behavior and decision-making, as well as misguided attention, most notably in cases of Attention Deficit/Hyperactivity Disorder (ADHD) (American Psychiatric Association, 2013).

A number of laboratories have provided evidence for a relationship between conditioned cue approach and impulsive behaviors (Lovic et al., 2011; Tomie et al., 1998). Conditioned cue approach is not easily extinguished (Williams and Williams, 1969) and these findings, in addition to assertions that conditioned cue approach expends

energy that is not actually necessary for the delivery reward, have led to the hypothesis that conditioned cue approach is itself a type of impulsive behavior (Monterosso and Ainslie, 1999). The term "impulsive behavior" can be used to describe an enormous variety of specific behaviors within varied cognitive domains; however, behaviors can be roughly categorized as either related to impulsive choice or impulsive action (Evenden, 1999; Dalley et al., 2011). Impulsive choices describe behaviors which lead to the immediate delivery of a smaller reward over a larger but delayed reward whereas impulsive action refers to situations in which animals fail to inhibit or control behavior, for example in cases of premature responding (Dalley et al., 2011). The two types of impulsivity can cluster within specific individuals, but that that is not always the case (Swann et al., 2002; Reynolds et al., 2006). Moreover, depending upon methodological considerations, participants may show signs of impulsivity in one behavioral assay but not another.

Rats (Richards et al., 1997), pigeons (Ainslie, 1974; Rodriguez and Lougue, 1988), and humans (Rodriguez and Logue, 1988) prefer small immediate rewards to larger delayed ones. Rats with orbitofrontal cortex, ventral striatum, or basolateral amygdala lesions are more likely to choose smaller immediate rewards (Cardinal et al., 2001; Rudebeck et al., 2006; Floresco et al., 2008). These findings indicate a role for each of these brain regions in impulse control. Risky choices, i.e. choosing a low-probability reward or choosing a reward that comes with a risk of an aversive event, is a phenomenon also proposed to be within the realm of "impulsivity." In rats, willingness to choose rewards associated with concomitant risk has been shown to be dependent upon dopaminergic circuitry, including responses within the prefrontal cortex and striatum (Simon et al., 2011; St Onge and Floresco, 2009; Treadway, 2012).

1.5. A ROLE FOR DOPAMINE

Given that both conditioned orienting behaviors and impulsive behaviors are reliant upon dopamine neurotransmission within the mesocorticolimbic and nigrostriatal pathways, it seems likely that individual differences in behavioral measures of orienting and impulsivity are indicative of underlying variation in dopaminergic function. This hypothesis is strengthened by recent work showing that rats more apt to show conditioned approach toward a lever predictive of food show enhanced dopamine transmission within the nucleus accumbens (Flagel et al., 2011; Saunders and Robinson, 2012). Individuals showing strong conditioned cue approach/engagement are believd to be more sensitive to the motivational power of the cues, making the cue itself an "object of desire" (Berridge, 2007). The incentive salience theory posits that these individuals have a sensitized mesocorticolimbic dopamine pathway that is more likely to attach greater motivation value to the reward-predictive cue, thus imbuing it with the power to elicit its own approach behavior (Berridge, 2012).

The human drug addiction literature consistently provides evidence for a relationship between substance abuse disorders, impulsive behavior, and dopamine sensitivity (Cloninger, 1986; Jentsch and Taylor, 1999; Tarter et al., 2007; Weafer & de Wit, 2013). Personality traits such as extraversion, positive affect, and strong motivation, traits believed to rely upon the mesolimbic dopamine system, relate to increased dopamine sensitivity and susceptibility to the addictive potential of drugs of abuse (Belcher et al., 2014; Depue & Collins, 1999; Depue, and Fu, 2013; Depue et al., 1994). These findings have led to the common assumption that individuals showing increased dopamine sensitivity are more sensitive to the dramatic increase in dopaminergic transmission elicited by drugs of abuse, thus leaving them more vulnerable to substance abuse disorders (Belcher et al., 2014).

Given the proposed relationships between conditioned cue approach, impulsivity, sensitivity to dopamine agonism and the overlap in these traits' associated brain regions, the goal of this dissertation was to explore the relationship between these traits and potential neural correlates within an animal model.

Chapter 2: Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus

2.1. ABSTRACT

When presented with a light cue followed by food, some rats simply approach the foodcup (Nonorienters), while others first orient to the light in addition to displaying the food cup approach behavior (Orienters). Cue-directed orienting may reflect enhanced attentional and/or emotional processing of the cue, suggesting divergent natures of cue-information processing in Orienters and Nonorienters. The current studies investigate how differences in cue processing might manifest in appetitive memory retrieval and updating using a paradigm developed to persistently attenuate fear responses (Retrieval-extinction paradigm; Monfils et al., 2009).

First, we examined whether the retrieval-extinction paradigm could attenuate appetitive responses in Orienters and Nonorienters. Both extinction given within the reconsolidation window was effective at persistently updating the initial appetitive memory in the Orienters, but not the Nonorienters. Since conditioned orienting is mediated by the amygdala central nucleus (CeA), our second experiment examined the CeA's role in the retrieval-extinction process. Bilateral CeA lesions interfered with the retrieval-extinction paradigm—did not prevent spontaneous recovery of food cup approach. Together, our studies demonstrate the critical role of conditioned orienting behavior and the CeA in updating appetitive memory during the reconsolidation window.

2.2. INTRODUCTION

When a neutral conditioned stimulus (CS) is paired with an unconditioned stimulus (US), animals often acquire cue-directed responses, for example, approaching/orienting to a light predictive of food (Brown and Jenkins, 1968; Holland, 1977). Under certain conditions, only a subset of animals acquires cue-directed behaviors (aka sign-tracking) in addition to, or at the cost of, developing US-directed behaviors (aka goal-tracking) that ultimately lead to the obtainment of a rewarding US. Cue-directed behaviors likely reflect enhanced attentional, emotional, and/or motivational processing of the cue (Holland, 1977; Robbins and Everitt, 1996; Cardinal et al., 2002) and represent how the cues themselves can acquire incentive value (Robinson and Berridge, 2001). Several brain regions/networks, including the amygdala and dopaminergic pathways, have been implicated in cue-directed behaviors (Gallagher et al., 1990; Parkinson et al., 2000, 2002; Lee et al., 2005, 2011; Mahler and Berridge, 2009; Flagel et al., 2011a,b). In particular, the amygdala central nucleus (CeA) and nigrostriatal circuitry are critical in mediating the conditioned orienting response (OR) directed to CSs paired with food, but are not involved in conditioned approach behavior to the food delivery site (Gallagher et al., 1990; Han et al, 1997; Lee et al, 2005; El-Amamy and Holland, 2006). These studies suggest a separate neural mechanism for cue-directed behaviors and that the nature of CS-information processing may be different in animals displaying robust conditioned cue-directed behaviors. What is not clear is how the presumably different natures of acquired CS-information influences memory extinction, retrieval and updating.

Extinction (repeated exposure to a CS that no longer predicts a US) gradually attenuates conditioned responses; however, this response attenuation is not permanent, and the conditioned responses can return in the form of renewal, reinstatement, or

spontaneous recovery (Pavlov, 1927; Rescorla and Heth, 1975; Bouton & Bolles, 1979; Robbins, 1990; Bouton, 2002). Thus, extinction does not generally modify the original CS-US association, but rather creates a separate CS-noUS memory that suppresses the original memory trace (Bouton, 2004). Recently, Monfils and colleagues (Monfils et al., 2009; Schiller et al., 2010) designed an extinction paradigm for fear conditioning in rats and humans that could potentially target the original CS-US association (see also Argen et al., 2012; Chan et al., 2010; Clem & Huganir, 2010; Rao-Ruiz et al., 2011). Standard extinction trials within 6-hrs of a single CS exposure blocked return of conditioned fear responses. The CS exposure presumably retrieved the original CS-US memory, which was then in a labile state needing to be re-consolidated (Nader et al., 2000; Nader, 2003; Tronson and Taylor, 2007). Thus, an extinction session after the cue-induced memory retrieval possibly updated the original CS-US association to a CS-noUS association. Others have also shown that this retrieval-extinction paradigm was effective in attenuating drug-seeking behaviors (Xue et al., 2012) in both humans and rats and in suppressing conditioned reinforcement in rats (Flavell et al., 2011).

In the current study, rats were categorized as Orienters and Nonorienters based on their display of conditioned responses during the acquisition phase. Orienters displayed robust conditioned orienting/rearing to the light CS in addition to acquiring conditioned food cup approach while Nonorienters acquired only the conditioned food cup approach. Because both groups showed comparable goal-tracking behavior (i.e., food cup approach), we termed them Orienters and Nonorienters (rather than sign- and goal-trackers) in order to more accurately describe their phenotypes. The first experiment examined whether the retrieval-extinction paradigm might be equally effective in blocking the return of Pavlovian appetitive responses directed to the CS (conditioned orienting/rearing response to the light) and to the US (conditioned food cup approach).

We further examined how individuals' predilections for the cue-directed orienting responses might manifest in memory retrieval and extinction. In the second experiment, we examined the role of the CeA in appetitive memory retrieval and extinction processes given the CeA's critical role in mediating conditioned OR.

2.3. MATERIALS AND METHODS

2.3.1. Subjects

Adult male Long-Evans rats (Harlan - Experiment 1, Charles-River – Experiment 2) weighing 250–275 g upon arrival were singly housed in a reverse 14-hr light/10-hr dark cycle, with the lights going off at 10 am. During acclimation, water and food were available ad libitum. One week after arrival to the colony (Experiment 1) or 7-10 days post-surgery (Experiment 2), rats were put on restricted feeding to reduce weight to 90% of their free-feeding body weight; this weight was maintained throughout the study. All experiments were conducted according to the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

2.3.2. Experimental Designs

2.3.2.1. Experiment 1: Effects of retrieval-extinction paradigm on conditioned OR and food cup approach.

In this experiment, extinction learning after memory retrieval was used to update the original appetitive memory. After animals were conditioned to light-food pairings, they received an extinction session within the reconsolidation window (i.e. a single CS

exposure before standard extinction trials). Then, spontaneous recovery rate was used to measure whether the original memory was updated.

Appetitive conditioning and testing took place in eight individual conditioning chambers that had aluminum sidewalls and ceiling, with clear acrylic front and back walls (30.5 cm W x 25.4 cm D x 30.5 cm H, Coulbourn Instruments). The floor was made of stainless steel rods (0.5 cm in diameter, spaced 1.0 cm apart). The food magazine was located on the right wall of the chamber, 2.5 cm above the floor. Nose-poke entry into the magazine was detected by an infrared beam at the opening. A 2-w white light was mounted 20 cm above the food-magazine and its illumination served as a CS signaling grain pellet delivery. The left wall was concave and had five ports with lights, which were not activated. Each chamber was enclosed in a light- and sound-attenuated box (58.4 cm x 61 cm x 45.7 cm) where the ventilation fan provided masking noise. Digital cameras were mounted within each box and images were recorded during behavioral training and testing.

Animals were first trained to eat a single grain pellet delivered to the magazine. A total of 30 pellets were delivered at a variable interval (averaging 60 s) over a 30-min session. After two pre-training sessions, all rats reliably retrieved grain pellets from the magazine. The first training session consisted of two parts. In order to habituate the unconditioned orienting response to light, the stimulus light was illuminated eight times, for 10-sec each time, without any food pellets being delivered to the magazine. Then, during the second half of the session, 8 trials of a 10-sec light presentation were followed by a food pellet delivery to the magazine. For the next three days of conditioning, sessions consisted of 16 light – food pairings with a variable intertrial interval (ITI) averaging 120 seconds. Extinction occurred 24 hours after the final training session. Prior to extinction, rats were pseudo-randomly divided into Retrieval and No Retrieval

groups in order for each group to have similar levels of conditioned food cup responding during acquisition. On the day of extinction, rats in the Retrieval group received one isolated CS presentation and were placed back in the home cage. After one hour in the home cage, they were returned to the conditioning boxes and received 17 CS-alone presentations. Rats in the No Retrieval group underwent a typical extinction session consisting of 18 CS-alone presentations, again with a variable ITI averaging 120 s.

Both groups received a test session 24 hours after extinction (Test One), which consisted of four CS presentations, given at variable intervals (average 120 s) without delivery of a grain pellet. Three weeks after this first test session, the rats were again tested with 4 presentations of the CS alone (Test Two). In summary, training (4 days), extinction, and Test One were completed in six consecutive days. After completing Test One, rats remained at 90% free feeding weight and were again tested 21 days after Test One.

2.3.2.2. Experiment 2: Role of CeA in appetitive memory updating within the reconsolidation window.

Prior to behavioral training, rats first received bilateral CeA lesions. They were anesthetized with isoflurane gas (Vet Equip) and placed in a stereotaxic frame (Kopf Instruments). Two sites per hemisphere were targeted; AP -2.0/-2.4, ML 4.2, DV -8.2. Rats in the lesion group received 0.2 μL infusion (per site) of 10 mg/mL ibotenic acid dissolved in a 0.1M phosphate buffered saline solution (PBS) (infused at 0.1 μL /min). Rats in the control group received a sham surgery consisting of either 0.2 μL infusion of PBS per site or lowering of the cannula into CeA with no infusion. Rats were allowed 7-10 days to recover before beginning food deprivation and training.

Training, retrieval-extinction, and test procedures for Experiment 3 were identical to those described in Experiment 1. However, for Experiment 3, the No Retrieval group

was subdivided into a context exposure group and a no context exposure group. Animals in the context exposure group were placed in the conditioning box one hour prior to extinction, but received no CS presentation. Animals in the no context exposure group remained in the home cage prior to extinction. As in Experiment 1, training (4 days), extinction, and Test One were completed in six consecutive days. After completing Test One, rats remained at 90% free feeding weight and were again tested 21 days after Test One.

Following behavioral testing, rats received an overdose of pentobarbital (86 mg/kg) and phenytoin (11 mg/kg) mix (Euthasol® by Virbac Animal Health) and were perfused transcardially with 0.9% saline followed by 4% Paraformaldehyde in 0.1 M phosphate buffer (PFA). Brains were removed, post-fixed, and cryoprotected overnight in a 20% sucrose PFA. Twenty-four hours later, brains were frozen in powdered dry ice and stored at -80 ºC. Brains were sliced on a freezing microtome and 30 µm sections were collected. In order to verify lesion size and placement, every fourth section was mounted on slides and Nissl-stained.

2.3.3. Behavioral analyses

Previous work has shown that when presented with a 10-sec light CS that predicts pellet delivery, rats typically show an orienting response towards the light during the first five seconds (CS1) and a food cup approach response during the last five seconds (CS2) (Holland, 1977). For all experiments, number of OR bouts were counted by a blind observer from DVD recordings of all training sessions. An OR was defined as a rearing response in which both forelimbs were lifted from the floor of the conditioning box, and did not include grooming behavior. To account for within-groups variation in baseline

orienting, we report the response difference in CS1 and pre-CS (the 5 seconds prior to the CS). Food cup approach is reported as bouts of nose-pokes into the magazine, as measured by the infrared beam. We report the difference in CS2 and pre-CS food cup responding.

2.3.4 Statistical analyses

For acquisition analyses of both experiments, orienting classification x trial repeated ANOVAs were conducted for orienting and food cup responses. For extinction analyses, orienting classification x retrieval condition x trial repeated ANOVA was conducted. When appropriate (Exp 1), it was followed with simple ANOVA within Orienters and Nonorienters for orienting response. For spontaneous recovery tests, orienting classification x retrieval condition x extinction/test days repeated ANOVA was conducted. When appropriate, it was followed up with separate ANOVAs with just Orienters or Nonorienters (Exp 1) or a priori comparison (Exp 2). When appropriate, the significant interaction effects were followed up with one-way ANOVA and then with Bonferroni test.

2.4. RESULTS

2.4.1. Experiment 1

2.4.1.1. Acquisition

During the conditioning sessions, in which the light cue was repeatedly paired with food, there was an overall acquisition of conditioned OR and food cup approach behavior. However, a subset of rats did not acquire conditioned OR. Thus, based on their

average number of OR bouts during the last eight trials of training, rats were divided into two groups. Rats scoring at or above the median number of OR bouts were classified as Orienters (n=26), while those rats that scored below the median score were classified as Nonorienters (n=22). As shown in Figure 2.1A, Orienters acquired conditioned OR to the light CS while Non-orienters did not show an increase in OR as training progressed. An orienting classification x trial block repeated ANOVA of OR showed a significant main effect of orienting classification, $F(1, 42) = 46.0$, $p < 0.0001$, a significant main effect of trial block, $F(6, 252) = 9.24$, $p < 0.001$, and significant interaction effects between the orienting classification and trial block, $F(6, 252) = 9.24$, $p < 0.001$. Importantly, Orienters and Nonorienters did not differ in their display of unconditioned OR. Both groups equally showed unconditioned OR at the beginning of the habituation trials and this unconditioned OR decreased over the course of the eight habituation trials: the average OR scores of the first 4 trials were 0.19 (Orienters) and 0.22 (Nonorienters), and the last 4 trials were 0.10 (Orienters) and 0.13 (Nonorienters). This was supported by a lack of main effect of orienting classification as well as orienting classification x trial interaction ($ps > 0.05$). Due to a video equipment malfunction, four rats were missing OR data from the eight habituation trials and first 8 trials of training and were excluded from analysis of OR data during habituation and training. The generally low levels of conditioned OR by Orienters (Fig 2.1A) partly reflect the nature of OR scoring and analyses procedures. Rats typically rear once towards the light within the first 5-sec but not at every trial, resulting the average score to be lower than one. In addition, even though it is not frequent, any baseline rearing during the 5-sec prior to the light onset has been subtracted, resulting in negative OR scores at some trials.

In contrast to conditioned OR acquisition, both Orienters and Nonorienters showed an increase in food cup responding as training progressed and there was no

difference in acquisition rate between these two groups (Figure 2.1B). An orienting classification X trial block repeated ANOVA of food cup responding showed only main effect of trial block, $F(6, 276) = 43.3$, $p < 0.001$.

2.4.1.2. Extinction

For an extinction session, animals were further divided into groups that received a single CS exposure an hour prior to standard extinction trials (Retrieval group) or only standard extinction trials (No Retrieval group). Thus, there were 4 groups of animals: Orienters-Retrieval (n=13), Orienters-No Retrieval (n=13), Nonorienters-Retrieval (n=11), and Nonorienters-No retrieval (n=11). As expected, Orienters showed more OR than Nonorienters, but the retrieval trial did not affect extinction rates (Fig 2.2A). An orienting classification x retrieval conditions x extinction trials repeated ANOVA supported this observation; there was a main effect of extinction trials, $F(8, 352) = 2.36$, $p < 0.05$ and a main effect of orienting classification, $F(1, 44) = 15.3$, $p < 0.0001$, but no interaction effects among orienting classification, retrieval conditions and/or extinction trials. Even though the interaction effect of orienting classification and extinction trials was not significant ($p=0.17$), the main extinction trial effect seemed to be driven by Nonorienters. Thus, we ran separate ANOVAs on Orienters and Nonorienters. The results show that the trial effect was only significant among Nonorienters, $F(8, 160) = 3.43$, $p=0.001$, but not among Orienters, $F(8, 192) = 0.98$, $p>0.4$. In terms of conditioned food cup responding (Figure 2.2B), all animals showed a reduction of food cup responding over the course of extinction trials, $F(8, 352) = 4.31$, $p < 0.05$, and there was no difference among the 4 groups, as shown by no main or interaction effects, $ps > 0.1$.

2.4.1.3 Test

Both 24 hours (Test One) and 21 days (Test Two) after extinction, rats were tested with 4 CS exposures. In order to determine whether there was spontaneous recovery of OR and food cup responding, the responses during the last 4 trials of extinction were compared to the responses during the test trials. Conditioned OR was observed in most of the animals regardless of extinction conditions (Figure 2.3A). As expected, Orienters generally showed higher levels of OR compared to Nonorienters. In support of this observation, an orienting classification x retrieval condition repeated ANOVA over extinction, Test 1, and Test 2 trials showed a main effect of orienting classification $F(1, 44) = 15.0$, $p < 0.0001$ and main effect of extinction-test days $F(2, 88) = 16.5$, $p < 0.001$, but no main effect of retrieval condition $F(1, 44) = 1.92$, $p > 0.1$. Furthermore, there was no interaction of orienting classification x extinction-test days, $F(2, 88) = 1.25$, $p>0.2$, no interaction of retrieval condition x extinction-test days, $F(2, 88) = 1.0$, $p>0.3$, and no three way interaction of orienting classification, retrieval condition and extinction-test days, $F(2, 88)=0.04$, $p>0.9$.

Conditioned food cup responding was different based on orienting classification and the retrieval condition (Fig 2.3B). Orienters in the No Retrieval group showed similarly increased food cup responding at both Test One and Test Two. By contrast, Orienters in the Retrieval group did not show much food cup responding at either test points. Food cup responding of Nonorienters in both Retrieval and No Retrieval groups increased during Test Two. In support of these observations, orienting classification x retrieval conditions repeated ANOVA over extinction, Test 1 and Test 2 trials showed a main effect of extinction-test days, $F (2, 88) = 10.2$, $p < 0.0001$, an interaction effect of orienting classification x extinction-test days, $F (2, 88) = 3.16$, $p < 0.05$, and an interaction effect of orienting classification x retrieval conditions, $F (1, 44) = 9.37$, $p <$

0.01. The interaction effects were further examined with follow-up analyses (i.e. retrieval condition x extinction/test days repeated ANOVA) conducted on Orienters and Nonorienters separately. Among Orienters, there was a main effect of retrieval condition, $F(1, 24) = 6.74$, $p < 0.05$, but no longer a significant main effect of test days, $F(2, 48) = 2.53$, $p > 0.05$. Among Nonorienters, there was only a main effect of test days, $F(2, 40) = 8.82$, $p = 0.001$ and no main effect of retrieval condition $F(1, 20) = 3.14$, $p > 0.05$. The results suggest that the retrieval-extinction paradigm reduced food cup responding among Orienters but not in Nonorienters.

2.4.2. Experiment 2

2.4.2.1. Histology

Twenty-four lesions were deemed acceptable. Lesions were rejected (n=10) if there was less than 30% damage to the medial CeA of either hemisphere or if there was extensive damage to surrounding areas such as the basolateral nucleus of the amygdala. Average bilateral lesion size was 65% damage of the entire CeA. Figure 2.4A and 2.4B show pictures of intact and lesioned CeA.

2.4.2.2. Acquisition

Rats with the CeA lesions were not expected to acquire conditioned OR. Thus, only rats in the sham surgery group were divided into Orienters and Nonorienters. This division provided three groups for analysis of training data: Lesion (n= 24), Orienter (n=18), and Nonorienter (n=18). As expected, Nonorienters as well as rats with good bilateral CeA lesions did not acquire conditioned OR. A group X trial block repeated ANOVA revealed a significant main effect of trial block, $F(6, 342) = 2.43$, $p < 0.05$, but also a significant group X trial block interaction, $F(12, 342) = 5.05$, $p < 0.001$. As seen in

Figure 2.4C, by the end of training Orienters displayed significantly higher conditioned OR when compared to Lesion rats and Nonorienters. A one-way ANOVA on the mean OR scores of the last 8 trials showed a main effect of groups, $F(2,57 = 27.8, p<0.001$, and a post-hoc Bonferroni test revealed that OR scores of Orienters were significantly higher from the ones of Nonorienters ($p<0.001$) and Lesion rats ($p<0.001$). As expected, there was no difference between Nonorienters and Lesion rats ($p>0.3$).

Regardless of the lesion/orienting classifications, all animals acquired the conditioned food cup response as training progressed and no differences in acquisition rates existed among these three groups. By the end of training, all reached the same levels of conditioned food cup approach (Figure 6C). A group X trial block repeated ANOVA showed only a main effect of trial block, $F (6, 324) = 29.78, p< 0.001$. There was neither a main effect of lesion/orienting classifications, $F(2, 57)=0.01, p=0.99$ nor an interaction effect of trial block by lesion/orienting classification, $F(12, 342) = 1.31, p>0.2$.

2.4.2.3. Extinction

At the end of training, Lesion rats, Orienters, and Nonorienters were further divided into the Retrieval and No Retrieval groups. Within the No Retrieval group, half of the rats were exposed to the context without the light CS while the others remained in their home cages. A lesion/orienting classification X retrieval condition (retrieval, context exposure, no context exposure) repeated ANOVA on food cup response revealed only a main effect of extinction trials, $F(17, 782) = 3.03, p<0.001$. Even though there was no main effect of retrieval condition, we did further analyses comparing just the context and no context exposure (i.e., orienting/lesion classification x context exposure repeated ANOVA with extinction trials) to make sure there was still no difference when these two

factors were directly compared. There was neither a main effect of context exposure, $F(1, 27) = 0.67$, $p > 0.4$, nor an interaction effect of context exposure by orienting/lesion classification, $F(2, 27) = 2.39$, $p > 0.1$. Therefore, the context and no context exposure groups were collapsed as the No Retrieval group. There were thus 6 groups; Lesion-Retrieval (n=11), Lesion-No Retrieval (n=13), Orienters-Retrieval (n=9), Orienters-No Retrieval (n=9), Nonorienters-Retrieval (n=7), Nonorienters-No Retrieval (n=11).

As expected, Orienters displayed more OR responses at the beginning of the extinction session compared to Nonorienters or Lesion rats (Figure 2.4D). However, the overall OR decreased throughout extinction and groups were not significantly different at the end of the session. A lesion/orienting classification X retrieval condition X trial repeated ANOVA confirmed a significant main effect of trial, $F(17, 918) = 2.23$, $p < 0.05$, as well as a lesion/orienting classification X trial interaction, $F(34, 918) = 1.62$, $p < 0.05$. One-way ANOVA on the mean OR scores of the first 2 trials showed a main effect of groups, $F(2,57) = 11.2$, $p<0.001$, and a post-hoc Bonferroni test revealed that OR scores of Orienters were significantly higher from the ones of Nonorienters ($p=0.001$) and Lesion rats ($p<0.001$). When the last 2 trials of OR scores were analyzed, there was no main effect of lesion/orienting classification, $F(2,57)=0.29$, $p>0.7$. In contrast to OR responding, the food cup approach did not differ among Orienters, Nonorienters, and Lesion rats (Figure 4E). A lesion/orienting classification X retrieval condition X trial repeated measures ANOVA revealed only a significant main effect of trial, $F(17, 918) = 3.27$, $p < 0.001$.

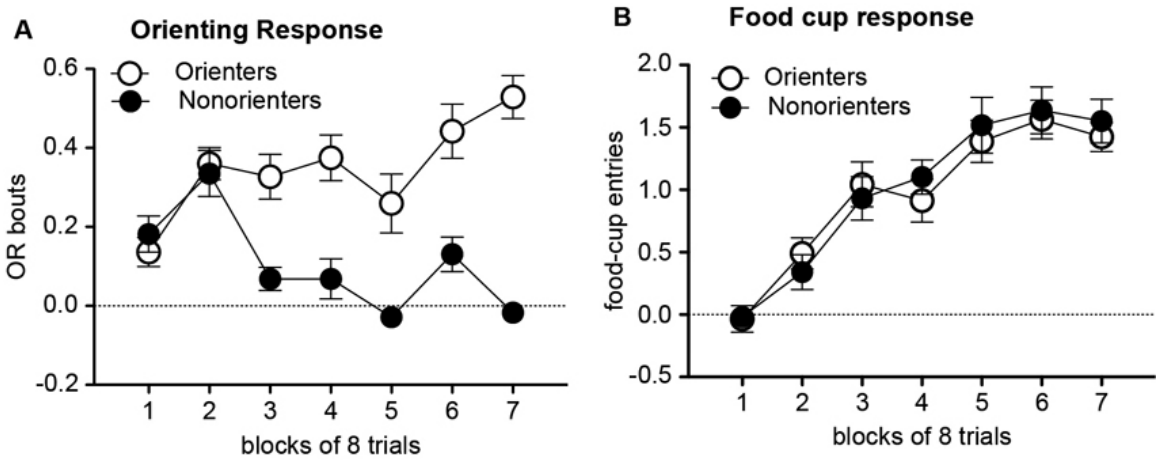
2.4.2.4. Test

Four rats (2 in the Orienter-No retrieval group, 1 in the Nonorienter-Retrieval group, and 1 in the Lesion-Retrieval group) did not receive light-CS exposures during

Test One. They were placed in the context, but a computer malfunction resulted in no light exposures. Because their behaviors did not differ from their cohorts in Test Two, their Test Two data were included. Thus, we ran orienting classification x retrieval conditions repeated ANOVA over extinction and Test 2 only. Including Test 1 as a repeated factor by eliminating those 4 rats did not change the results.

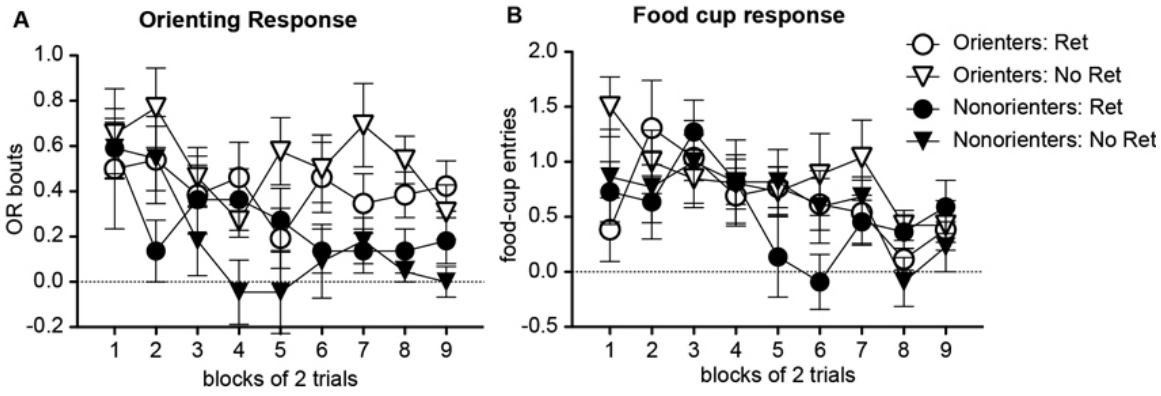
Conditioned OR was observed in most of the animals regardless of retrieval condition or orienting/lesion classifications. There was only a main effect of extinction/test days, $F(1, 54) = 16.14$, $p < 0.001$ (see Table 2.1 for the OR data). Similar results were found with the food cup responses. There was only a main effect of extinction/test days, $F(1, 54) = 21.7$, $p < 0.001$ (Figure 2.5). Even though there were no significant interaction effects, we conducted a priori planned comparisons to confirm that the retrieval-extinction paradigm was still effective at keeping the food cup response low for Orienters when tested 3 weeks after extinction. Paired t-tests between extinction and Test 2 for the Retrieval condition in each orienting/lesion classified groups confirmed no significant effect among Orienters, $t(8)=0.61$, $p>0.5$, but significant effects among Nonorienters, $t(6)=3.29$, $p=0.0167$, and Lesion rats, $t(10)=2.95$, $p=0.014$ after correcting for multiple comparisons (significant p value at 0.0167).

Figure 2.1. Acquisition of orienting and food cup approach



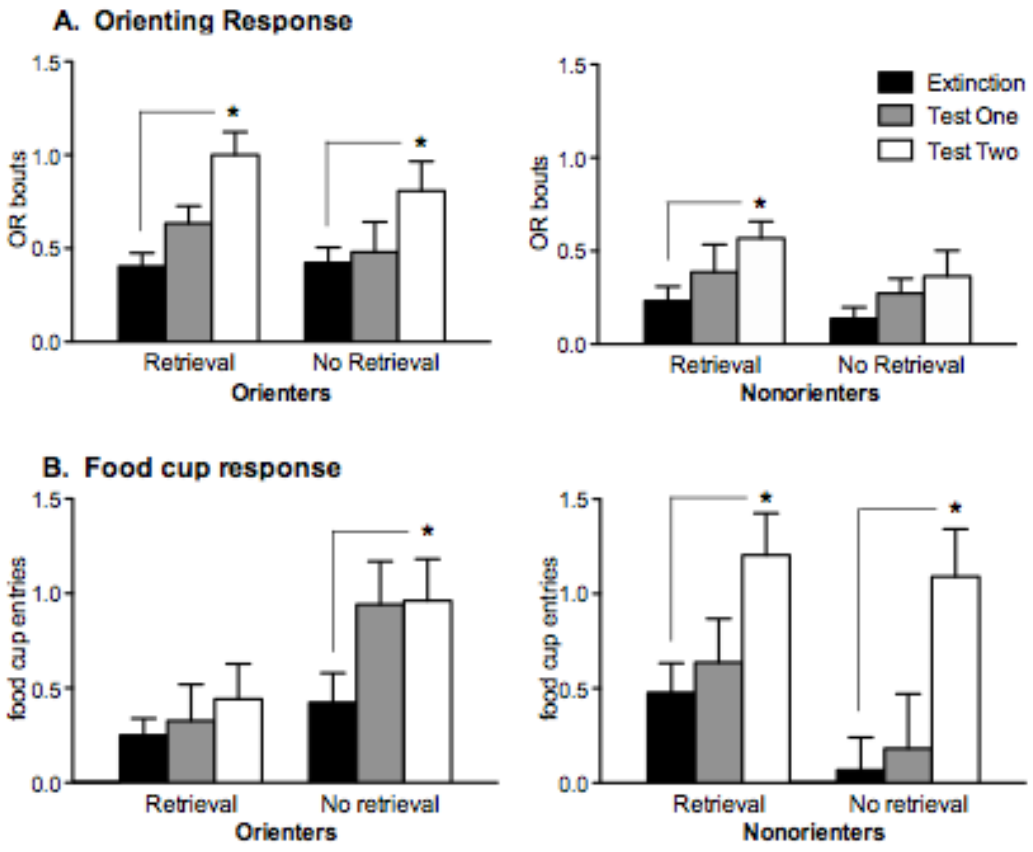
Mean (±SEM) OR (A) and food-cup response (B) during training. OR bouts were measured during the first 5 s of each CS and food-cup entries were measured during the last 5 s CS period. The values shown are elevation scores, calculated by subtracting pre-CS baseline responding from responding during the CS. Orienters, but not Nonorienters, acquired conditioned OR to the light CS, $p < 0.0001$ (A). In contrast, both Orienters and Nonorienters acquired conditioned food-cup responding (B).

Figure 2.2. Extinction of the orienting response and food cup response



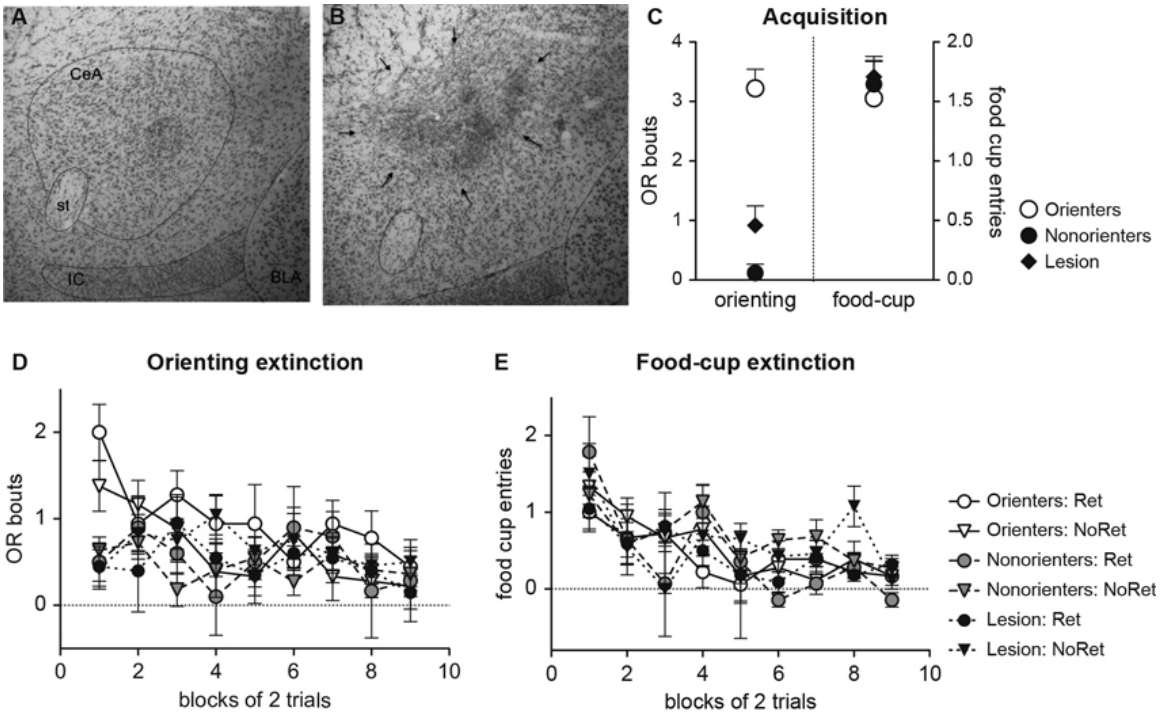
Mean (±SEM) OR (A) and food cup response (B) during extinction. Orienters and Nonorienters refer to the animals that showed robust and no conditioned orienting, respectively, during conditioning phase. Ret refers to the extinction condition, in which a single CS was presented prior to regular extinction trials while No Ret refers to the regular extinction trials without a prior CS presentation. Orienters showed more OR than Nonorienters (A). There was no difference in food-cup responding among four groups, and all showed comparable extinction rates (B).

Figure 2.3. Recovery of the orienting and food cup responses



Mean (±SEM) OR (A) and food cup response (B) for Orienters (left panels) and Nonorienters (right panels). The values are responses during the last four CS alone presentations in extinction session, four CS alone presentation 24 h (test 1) and 21 days (test 2) after extinction. A single CS presentation 1 h prior to extinction trials (retrieval condition) blocked return of spontaneous food-cup response only in Orienters.

Figure 2.4. Acquisition and extinction of orienting and food cup approach



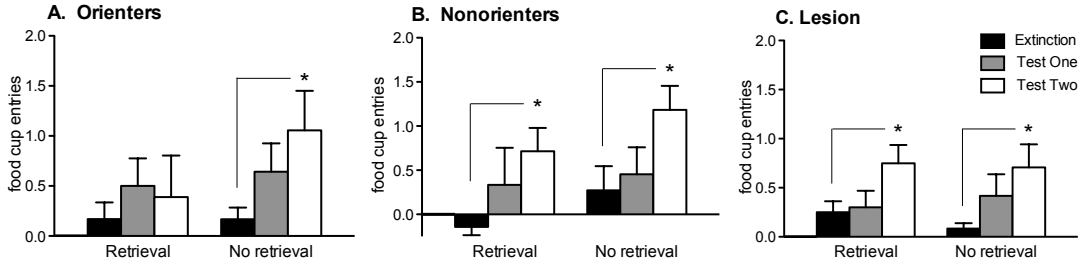
Representative photomicrographs of the amygdala region from the animals with sham lesion **(A)** and ibotenic acid lesion **(B)**. Central amygdala (CeA), stria terminalis (ST), intercalated nucleus (IC), and BLA are highlighted. Average lesion size was 65% CeA damage, and rats with significant BLA damage were excluded. **(C)** Mean (±SEM) OR and food cup response during the last eight trials of training for Orienters, Nonorienters, and Lesion rats. Animals with CeA lesions showed minimal conditioned OR, but still showed intact conditioned food-cup response. **(D and E)** Mean (±SEM) OR and food cup response during extinction. Orienters showed more OR than Nonorienters and CeA Lesioned rats at the beginning but at the end. There was no difference in food-cup responding among six groups, and all showed comparable extinction rates.

Table 2.1. Recovery of orienting response

		Extinction	Test 1	Test 2
Orienters	Ret	0.44(0.23)	1.56(0.30)	1.17(0.30)
	No Ret	0.22(0.12)	1.14(0.43)	0.89(0.25)
Nonorienters	Ret	0.33(0.56)	1.00(0.39)	1.07(0.43)
	No Ret	0.36(0.19)	0.95(0.32)	1.45(0.18)
Lesion	Ret	0.18(0.21)	0.90(0.24)	0.85(0.22)
	No Ret	0.62(0.16)	1.04(0.18)	0.63(0.25)

Mean (±SEM) orienting response during the last 2 trials of extinction and the first two trials of test done at 24-h (Test 1) and 21-days (Test 2) after extinction.

Figure 2.5. Recovery of food cup approach



Mean (±SEM) food-cup responding during extinction and tests both 24-h (Test 1) and 21 days (Test 2) after extinction. The values are responses during the last two CS alone presentations of the extinction session, and the first two CS alone presentations during Test 1 and Test 2. Orienters in the retrieval condition are the only animals not showing spontaneous recovery of conditioned food-cup response.

2.5. DISCUSSION

The current studies highlight the role of conditioned OR in cue processing, specifically in cue-associated memory retrieval and updating. Experiment 1 showed that extinction within the reconsolidation window was effective at persistently reducing conditioned food cup approach only in those rats that showed robust conditioned OR during the acquisition phase. These results suggest that the differences in the display of conditioned OR reflect fundamental differences in stimulus encoding, memory retrieval and updating. Also, Experiment 2 suggests that the CeA, known to be necessary for the acquisition of conditioned OR, is critical for the retrieval-extinction paradigm to effectively block return of conditioned food cup behavior.

2.5.1. Robust effects of the retrieval-extinction paradigm in diverse procedures

It should be noted that the attenuation of conditioned food cup response, following the retrieval-extinction paradigm was replicated in these experiments despite several major differences between the original Monfils (2009) work and the current study. The differences included valence of the US (shock vs. food pellet), modality of the CS (tone vs. light), number of CS-US pairings (3 vs. 56), rat strain (Sprague-Dawley vs. Long-Evans), and circadian rhythm (testing in light vs. dark cycle). As was the case in Monfils et al. (2009), the current study also showed that the retrieval-extinction paradigm relied on exposure to the specific CS and not on general exposure to the context. The context exposure effect was directly tested in Experiment 2 among animals in the No Retrieval group; one subgroup was exposed to the context without CS presentation while the other group remained in the home cage. Equivalent spontaneous recovery was observed in both groups. Thus, the current study suggests that the retrieval-

extinction paradigm can be effective in updating appetitive memory. In fact, other recent studies have reported that the retrieval-extinction paradigm was effective in a variety of appetitive settings. For example, extinction after drug-associated cue presentation prevented drug-seeking behaviors in rats and drug craving in humans (Xue et al., 2012). In another study (Flavell et al., 2011), rats did not acquire conditioned reinforcement with a food-associated light cue that was subjected to the retrieval-extinction paradigm. However, unlike earlier findings, our results showed that the retrieval-extinction paradigm worked only in a subset of animals (Orienters). Moreover, unlike conditioned food cup approach behavior, conditioned OR was not affected by the retrieval-extinction paradigm; Orienters continued to show conditioned OR during the tests.

2.5.2. Specific effects of the retrieval-extinction paradigm on food cup response

Although both OR and food cup approach behavior are reflective of CS-US associative strength, conditioned OR is thought to reflect attentional processing in particular (Holland, 1977; Holland and Gallagher, 1999). In support, various studies have shown independent neural processing of these two conditioned responses. Conditioned OR, but not conditioned food cup response, relies on the CeA-nigral dopamine system (Han et al., 1997; Lee et al., 2005; El-Amamy & Holland, 2007), which has also been implicated in several behavioral tasks designed to measure attentional processing (Lee et al., 2006, 2007, 2008, 2009). Interestingly, the CeA is only required during the acquisition of conditioned OR and is unnecessary for the expression of fully acquired conditioned OR (McDannald et al., 2004). In contrast, the nigro-dorsolateral striatal circuitry is needed to express conditioned OR (Han et al., 1997; El-Amamy and Holland, 2006), suggesting a habit-like process of fully conditioned OR. Thus, extinction

during the reconsolidation window may not target fully conditioned OR that relies on the dorsolateral striatum for expression. The neural circuitry underlying the conditioned food cup response is unknown; however, the basolateral nucleus of the amygdala (BLA), but not the CeA, is known to play an important role in encoding and representing reinforcement value of the CS (Hatfield et al., 1996). In particular, the BLA and its connections with the orbitofrontal cortex are important for updating the current value of a specific CS (Gallagher et al., 1999; Schoenbaum et al., 1999, 2003 a&b). Thus, different neural circuitries contribute to different processes engaged in appetitive conditioning (Holland and Gallagher, 1999). The retrieval-extinction paradigm, which aims to update the original CS-US association to a CS-no US association might be more effective at targeting the neural process for encoding and updating CS value rather than the process important for regulating attention to CS. Interestingly, in Xue et al study (2012), the retrieval-extinction paradigm influenced protein kinase Mζ expression in the BLA, but not in the CeA.

It should be pointed out that both Nonorienters and rats with CeA lesions showed orienting responses comparable to Orienters during the test days (see Table 2.1). Both at the end of acquisition phase (Figure 2.4C) and at the beginning of extinction session (Figure 2.4D), Nonorienters and CeA lesioned rats showed significantly fewer orienting responses as compared to Orienters, as expected. However, during the habituation period when the light CS is presented without food, all three groups of rats displayed comparable unconditioned orienting responses: overall OR counts over 8 trials were 2.4 (Orienters), 2.5 (Nonorienters), and 2.2 (CeA lesion). In accord, previous work (and the current study) has repeatedly shown neural and behavioral dissociations between unconditioned and conditioned orienting (Gallagher et al., 1990; Lee et al., 2005, 2011). Thus, one possibility is that the return of orienting seen during the tests might partly

reflect unconditioned orienting. Our interpretation of this finding is limited in the current form and further investigation is needed.

2.5.3. Individual variations in the display of conditioned orienting and memory updating

Even though the retrieval-extinction did not influence conditioned OR, the effectiveness of this paradigm at persistently reducing conditioned food cup behavior was influenced by the animals' propensity to display conditioned OR. Others have shown individual differences in the display of cue-approach behavior, also termed sign-tracking (see Flagel et al., 2009 for review) and reported behavioral and physiological differences seen in sign-trackers. For example, different monoamine activities in mesolimbic system (Flagel et al., 2007, 2010, 2011; Tomie et al., 2000), elevated corticosterone levels (Tomie et al., 2000), enhanced cocaine-induced psychomotor sensitization (Flagel et al., 2008), and high impulsivity (Tomie et al., 1998; but see Lovic et al., 2011) have been reported in sign-trackers. Our unpublished work also suggests that Orienters make more impulsive decisions and show enhanced 50-kHz ultrasonic vocalization in response to amphetamine. While some specific circuitries remain unknown, dopamine neurotransmission appears to be involved in all forms of sign-tracking behaviors. In particular, Flagel et al. (2011b) showed an interaction of dopamine and cue-approach behavior: dopamine release in the nucleus accumbens following the CS was associated with animals showing prepotent sign-tracking behavior and intact dopamine function was necessary for the acquisition of sign-tracking. These data suggest that animals with a natural tendency to develop cue-approach behavior encode and process stimulus information differently from animals that do not show robust cue-approach behavior.

In the current studies, presumably enhanced attention to the CS (as measured by heightened conditioned OR) may allow for complete retrieval of the original CS-US memory, subsequently making that memory more apt for updating during extinction or new learning. Given that the CeA-nigral dopamine circuitry is essential for the acquisition of conditioned OR (Lee et al., 2005; El-Amamy and Holland, 2006), rats that show a natural tendency to develop a prepotent conditioned OR may have enhanced CeA-nigral dopamine function. Under normal extinction trials, the CeA-nigral circuitry's role may not be as important, as typical extinction (or new learning) most likely does not rely upon retrieval of a previously acquired CS-US memory. However, enhanced CeA-nigral dopamine function may aid extinction during reconsolidation by enhancing cue-induced retrieval of CS-US associative memory and updating it to a CS-no US memory. This view is supported by findings from Experiment 2, as rats with CeA lesion showed food cup responding 3 weeks following retrieval-extinction, an indication that they were unable to permanently update the value of the CS. A future study will be needed to address whether the intact CeA function is necessary at the time of appetitive acquisition and/or during memory retrieval-extinction.

We also observed a trend in differences of food cup approach between Orienters and Nonorienters when they were tested a day after extinction: Orienters showed substantial conditioned food cup approach, which was not evident among Nonorienters (test 1 data of No Retrieval group in Fig 2.1B). The observed conditioned food cup approach in Orienters-No Retrieval group during test 1 was only marginally significant compared to its own food cup behavior seen at the end of extinction ($p=0.094$), but was significantly different (without correcting for multiple comparisons) from the food cup behavior seen in Nonorienters-No Retrieval group at test 1 ($p=0.047$). However, this observation was not replicated in Experiment 2, questioning the consistency of this

particular phenomenon observed between Orienters and Nonorienters. Nonetheless, the retrieval-extinction paradigm was effective at keeping the conditioned food cup approach low at both test 1 and 2 for Orienters. More work is needed to examine the potential orienting phenotypic differences in maintenance of extinguished food cup behavior, which can have implications in the interpretations of how the retrieval-extinction paradigm reduces food cup behavior persistently.

2.5.4. Mechanisms of the retrieval-extinction paradigm

Even though the current study is limited in providing mechanistic explanation, it contributes to our understanding of the retrieval-extinction paradigm on memory maintenance and opens the door for many follow-up experiments to be conducted, in the appetitive as well as fear fields. One possible explanation of the current results is that the retrieval-extinction manipulation works via memory updating mechanism. In Monfils' 2009 work, GluR1 phosphorylation in the lateral nucleus of the amygdala was increased following a single CS presentation, but returned to baseline levels after the administration of a second CS one hour, but not 3 minutes, after the first. Other studies (Rao-Ruiz et al., 2011; Clem and Huganir, 2010) also provided evidence consistent with the results and mechanistic explanation Monfils provided in 2009 and 2010 in the follow up study in humans (Schiller et al., 2010).

Recently, Baker et al. (2013) showed that a single CS presentation either before or after a standard extinction session (i.e., retrieval+extinction or extinction+retrieval) essentially produced the same effect. They suggested that these two manipulations were driven by the same mechanism; that is some form of facilitation and/or strengthening of extinction would be occurring due to the spacing of the stimuli. We believe that the

retrieval+extinction and extinction+retrieval, though they yield similar behavioral outcomes, are likely to operate through different mechanisms—the retrieval-extinction is due to an updating during reconsolidation, and the extinction+retrieval is due to extinction facilitation/strengthening. The study by Baker et al. (2013) does not allow for a distinction in mechanisms, since they only tested behavior (freezing). Published data from our lab as well as others generally point to the latter interpretation of memory updating (Clem and Huganir, 2010; Monfils et al., 2009; Rao-Ruiz et al., 2011). Nevertheless, Baker et al.'s approach is an interesting one and contributes to the field by introducing potential factors that can influence extinction and memory updating. For example, the Baker et al. study found the retrieval-extinction effect in young adolescent rats while their earlier study did not find the retrieval-extinction effect in adult rats (Chan et al., 2010). Our current study tried to address whether the retrieval+extinction effect on fear conditioning was generalizable to another form of learning, but also aimed to understand some of the boundary conditions that may be contributing to the variability in reported effects from various groups.

2.5.5. Implications

Work investigating how CSs elicit and maintain certain conditioned responses is important in delineating the psychological processes and neural mechanisms that contribute to drug addiction. Accumulating evidence suggests an important role of associative learning processes in drug addiction, in which the environmental cues become associated with reinforcing effects of a drug and later induce a vulnerable state of drug craving and elicit drug-seeking behaviors (Everitt at al., 1999; Weiss et al., 2001; Wise, 2004; Hyman et al., 2006; Robbins et al., 2008; Robinson and Berridge, 2008; Belin et

al., 2009). Thus, weakening or undoing the cue-drug association can potentially prevent drug relapse (Taylor et al., 2009). In fact, Xue and colleagues (2012) showed that the retrieval-extinction paradigm was effective in reducing drug craving and relapse. However, they reported that the drug seeking behavior was only reduced, and not completely blocked, in some cases. Our study suggests that individual differences in cue-directed behavior may affect memory retrieval and updating of CS-associated memory differently. Thus, treatments for drug addiction based on the retrieval-extinction paradigm might work more effectively in a subset of populations. Further studies will be necessary to understand if individual differences in processing discrete CS-associated memory can be used effectively to target drug-associated memory.

Chapter 3: Appetitive behavioral traits and stimulus intensity influence maintenance of conditioned fear

The work reported in Chapter 3 was completed with equal contributions from M.E. Olshavsky and C.E. Jones.

3.1. ABSTRACT

Individual differences in appetitive learning have long been reported, and generally divide into two classes of responses: cue- vs. reward-directed. The influence of cue- vs. reward-directed phenotypes on aversive cue processing, is less well understood. In the current study, we first categorized rats based on their predominant cue-directed orienting responses during appetitive pavlovian conditioning. Then, we investigated the effect of phenotype on the latency to exit a familiar dark environment and enter an unfamiliar illuminated open field. Next, we examined whether the two phenotypes responded differently to a reconsolidation updating manipulation (retrieval+extinction) after fear conditioning. We report that the rats with a cue-directed ("orienting") phenotype differentially respond to the open field, and also to fear conditioning, depending on US-intensity. In addition, our findings suggest that, regardless of appetitive phenotype or shock intensity, extinction within the reconsolidation window prevents spontaneous recovery of fear.

3.2. INTRODUCTION

When pairing a conditioned stimulus (CS) with a biologically significant event such as food (unconditioned stimulus, US), rats develop conditioned responses (CR) that are CS-directed (aka sign-tracking) and/or US-directed (aka goal-tracking), and

individual differences exist in the predominance of each response. In the case of light-food pairings, some rats develop both CS- and US-directed responses, that is, they orient/rear towards the light cue and approach the site of food delivery, while other rats develop only the food cup approach behavior. Because both groups exhibit the goal-tracking response (approach to the food cup) and only a subset develops a sign-tracking response (orienting to the light CS), we characterize these groups based on their conditioned orienting response to the CS and classify them as Nonorienters and Orienters, respectively.

Numerous reports, including our own, have indicated that these two phenotypes differ in measures of risky decision making, delay discounting, novelty preference, dopaminergic response to cues, and response to drug exposure (Flagel et al., 2011; Lovic et al., 2011; Olshavsky et al., 2012; Yager and Robinson, 2012). Orienters and Non-orienters also behave differently in their susceptibility to appetitive memory updating (Thompson et al., 2011). Effectively, using a procedure based on a paradigm developed in fear conditioning experiments (Monfils et al., 2009; Schiller et al., 2010) Olshavsky et al., (this issue) observed that rats receiving a retrieval trial prior to extinction showed attenuated conditioned responding during tests for spontaneous recovery (Olshavsky et al., 2013) but that this effect was dependent on whether the rats were Orienters or Nonorienters—only Orienters showed attenuation of conditioned responding after the retrieval-extinction procedure. This result is particularly important in light of the fact that many (Clem and Huganir, 2010; Schiller et al., 2010; Rao-Ruiz et al., 2011) but not all (Chan et al., 2010) labs have observed the persistent fear memory updating described in Monfils et al. (2009), prompting a need to investigate the boundary conditions that surround this form of memory updating. To this effect, for the present study we first classified rats as either Orienters or Nonorienters based upon their expression of either

CS-directed or US-directed responses during light-food pairings, we then compared their behaviors within an open field task, then tested whether expression of conditioned fear differs in rats that show robust cue-oriented responding and those that do not, and finally, examined whether fear memory could be persistently attenuated in those groups using the retrieval+extinction paradigm (Monfils et al., 2009).

3.3. MATERIALS AND METHODS

3.3.1. Subjects

Sixty-six Long-Evans male rats (250-275 g upon arrival, Charles River Laboratories) were used. Rats were maintained on a 12-hr regular light-dark cycle with lights on at 7am. For the open field and appetitive conditioning portions of the experiment, rats were maintained at 90% free-feeding weight; water was available ad libitum. During fear conditioning procedures, food and water were both provided ad libitum. All experiments were conducted according to *the National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Initially, rats were trained to retrieve food pellets from a food cup located within an appetitive conditioning chamber. Eight individual conditioning chambers (30.5 W x 25.4 D x 30.5 H in cm, Coulbourn Instruments, Allentown, PA) with aluminum sidewalls and ceiling, clear acrylic front and back walls and stainless steel rod floors (rods 0.5 cm in diameter, spaced 1.0 cm apart) comprised the appetitive conditioning context. A wall-mounted magazine delivered grain pellets (Test Diet, 45 mg) to a recessed food cup mounted 2.5 cm above the floor. Each chamber was enclosed in a light- and sound-attenuated box (58.4 x 61 x 45.7 cm); a ventilation fan provided masking noise. A video

camera was mounted within each box and images were recorded during behavioral training. During the initial food cup training a total of 30 pellets were delivered to the food cup at a variable intertrial interval (ITI) averaging 60 s over a 30-min session. After one session, all rats reliably retrieved the grain pellets.

3.3.2 Open field

After food cup training, both rats' latency to enter an illuminated open field and their preference for the illuminated open field vs a familiar dark compartment were assessed. Two open field chambers consisting of white acrylic floors surrounded on all sides by clear acrylic walls were used (43.2 W x 43.2 D x 30.5 H in cm). On day 1, rats were restricted to an opaque black insert (43.3 W x 21.6 D x 30.5 H in cm) for 10 minutes. The following day rats were initially placed within the black insert, but were free to exit into the illuminated portion of the open field and had 10 minutes of free access to both sides. Activity in both sides of the field was detected by infrared beam motion detectors.

3.3.3. Appetitive conditioning

Forty-eight hours after completing the open field test, rats began appetitive conditioning. The first day of appetitive conditioning consisted of two parts. In order to habituate the unconditioned orienting response to light, the stimulus light (2-Watt white light mounted 20 cm above the magazine) was illuminated eight times, for 10 s each time, without any food pellets being delivered to the magazine. Then, during the second half of the session, 10 s light-CS illuminations were followed by grain pellet delivery into

the food cup. For the next three days of conditioning, sessions consisted of 16 light–food pairings with a variable ITI averaging 120 +/- 50 seconds.

Nosepoke to the food cup was detected by an infrared beam at the opening, while orienting behavior was scored by a blind observer from DVD recordings of sessions. Orienting measures were directly adapted from the ones used by Holland and colleagues (Gallagher et al., 1990; Lee et al., 2005, 2010, 2011). Even though the light-CS was a localized cue, it still provided diffuse illumination of the entire chamber. Thus, an orienting response was defined as any rearing response in which both forepaws were lifted from the floor of the training box, but did not include grooming behavior. For each light-food trial, behavior was sampled at every 1.25 sec resulting in 12 observations: 4 times during the 5 seconds immediately preceding the onset of the CS (preCS), 4 times during the first 5 seconds of the CS (CS1), and 4 times during the last 5 seconds of the CS (CS2). Because orienting response and food cup approach occur predominantly during CS1 and CS2, respectively (Holland, 1977), we report orienting response from CS1 and food cup approach behavior from CS2. Their behaviors during preCS are subtracted to account for any baseline differences.

3.3.4. Fear conditioning

Following appetitive training, rats were transferred to a new colony and after a 3-5 day of acclimation, all rats were fear conditioned in a second context. All remaining procedures (fear conditioning, long-term memory test, and the test for spontaneous recovery) were conducted in this second context. Rats were fear conditioned in chambers equipped with two metal walls, two clear plexi-glass walls, and stainless-steel rod floors connected to a shock generator (Coulbourn Instruments, Allentown, PA). Each

conditioning chamber was enclosed in an acoustic isolation box (Coulbourn Instruments) and lit with a red house light. Behavior was recorded with digital cameras mounted on the top of each unit. Stimulus delivery was controlled using Freeze Frame software (Coulbourn Instruments). The CS used for fear conditioning was a 20-sec tone (5 kHz, 80dB). The US was either a 0.7 or 1.0 mA footshock 500 ms in duration. Orienters and Nonorienters, as determined by the orienting response during the last eight trials of appetitive training, were divided into two shock intensity groups for fear conditioning (0.7 and 1.0 mA). On the fear-conditioning day, after a 2-minute habituation period, all rats received three 20-second presentations of the tone CS (variable ITI = 120 s), each co-terminating with either a 0.7 or 1.0 mA foot-shock. An experimenter blind to group assignment scored freezing behavior manually from video recorded during each session. Freezing was defined as the absence of any movements, excluding those required for respiration. The total number of seconds spent freezing throughout the CS presentation was expressed as a percentage of CS duration.

Twenty-four hours after fear conditioning, all subjects underwent either extinction (ext only) or retrieval+extinction (ret+ext). For the extinction session, rats were placed in the fear-conditioned context and exposed to 19 non-reinforced presentations of the tone CS (variable ITI = 120 s). A subset of these rats (n=21 out of 37) in the extinction only group were placed in the context 10 minutes prior to the extinction session but received no CS presentations. Context-exposed and non-context-exposed rats from the No Retrieval groups were not significantly different and these groups were collapsed for the remainder of analyses. Rats in the ret+ext group were first exposed to a single CS presentation in the fear-conditioned context, returned to the home-cage for 10 minutes, and then returned to the same context for the remaining 18 extinction trials. This resulted in eight groups for analysis - Orienter 0.7 mA ret+ext n=8; Orienter 0.7 mA ext only n=9;

Nonorienter 0.7 mA ret+ext n=9; Nonorienter 0.7 mA ext only =7; Orienter 1.0 mA ret+ext n=8; Orienter 1.0 mA ext only n=9; Nonorienter 1.0 mA ret+ext n=9; Nonorienter 1.0 mA ext only n=8.

3.4. RESULTS

3.4.1. Appetitive conditioning

Based on their average number of orienting bouts during the last eight trials of training, rats were divided into two groups. Rats scoring at or above the median (0.38 bouts/trial) were classified as Orienters (n= 34), while those rats that scored below the median were classified as Nonorienters (n= 32). The mean conditioned orienting levels, 0.85±0.07 and -0.01±0.04, were significantly different between Orienters and Nonorienteres, respectively, $t(64)=9.84$, $p<0.0001$ (Figure 3.2A). Groups of rats, however, did not differ in displaying conditioned food cup approach (Figure 3.2B). Furthermore, the groups did not differ in unconditioned orienting response during the first 8 trials, in which light was presented without any food: Mean orienting bouts during those trials were 0.36 for Orienters and 0.35 for Nonorienters ($p=0.91$) (data not shown).

3.4.2. Open field

Analysis of data collected during the dark-light open field task indicated that Orienters exited the dark insert (and entered the illuminated field) more quickly than Nonorienters, $t(64)=1.98$, $p=0.05$ (Figure 3.2C). There was also a trend for Orienters to spend more time in the illuminated field than Nonorienters, $t(64) = 1.85$, $p=0.07$. These results cannot be attributed to a difference in general activity levels, as the ambulatory distance traveled of the two groups were comparable, $t(64)=0.91$, $p=0.37$ (Fig.2C insert).

3.4.3. Fear Conditioning

Freezing during the fear conditioning session was analyzed using mixed factor ANOVAs with fear conditioning cue (3 cues total) as the repeated measure and orienting classification (Orienter or Nonorienters) and shock intensity (0.7mA or 1.0mA) as the between subjects factors. There was a significant within-subjects effect of fear conditioning cue, $F(2,116)=391.58$, $p<.001$, indicating that rats froze significantly more towards the end of the fear conditioning session than at the beginning. Additionally, overall rats froze significantly more throughout conditioning to the 1.0 mA than the 0.7 mA. In addition the Orienters and Nonorienter were differentially affected by shock intensity. There was a significant fear conditioning cue x shock intensity interaction, $F(2,116)=3.74$, $p=0.027$ as well as an overall main effect of both orienting classification, $F(1,58)=4.17$, $p=0.046$, and shock intensity, $F(1,58)=5.36$, $p=0.024$. Follow up ANOVAs for each shock intensity revealed that for the 0.7mA fear conditioning group (Figure 3.3A), there were no differences in freezing levels during acquisition between the Orienters (n=15) and Nonorienters (n=14), $F(1,27)=0.49$, $p=0.49$. However, rats classified as Orienters who were fear conditioned to the 1.0 mA shock (n=17) froze significantly less than rats classified as Nonorienters (n=16) evidenced by an overall main effect of orienting on freezing levels during the fear conditioning session, $F(1,31)=4.57$, $p=0.041$ (Figure 3.3B). However, a comparison of the mean freezing of Orienters and Nonorienters in the 1.0 mA group revealed that the groups were not significantly different during the last trial of conditioning.

3.4.4. Contextual fear

Contextual fear was measured by scoring freezing during a 20 second sample within the first 2 minutes that the rat was placed in the fear conditioning context the day after fear conditioning. For rats that received a CS retrieval, freezing to the context was measured in the 20 seconds immediately preceding the CS onset. In the ext only group, rats that received a context exposure only, freezing to the context was measured for 20 seconds at the same time point as the retrieval group. In the subset of animals that did not receive a context exposure, context freezing was measured in the 20 seconds preceding the first CS of extinction. All of these measurements took place at the same time point during the rat's first exposure to the fear conditioning context. A 2x2 ANOVA with orienting classification and shock intensity as the factors revealed a significant main effect of shock intensity, $F(1,62)=15.96$; $p<0.001$, no main effect of orienting classification, $F(1,62)=1.90$; $p=0.173$, and an orienting classification X shock intensity interaction, $F(1,62)=7.73$; $p=0.007$. Follow up t-tests revealed that there were no significant differences between Orienters and Nonorienters after conditioning to a 0.7mA shock, $t(31)=1.74$; $p=0.092$, and overall contextual freezing levels were very low (<10%) as seen in Figure 3.3C. However, after conditioning to a 1.0mA footshock, Nonorienters showed significantly more freezing to the context than Orienters, $t(31)=2.27$; $p=0.03$ (Fig 3D).

3.4.5. Extinction/retrieval+extinction

Given the differences between Orienters and Nonorienters in freezing during the 1.0 mA fear conditioning session, we compared the mean of the first four trials of extinction and tested whether our groups differed in their fear conditioning retention. Neither orienting classification, shock level, nor retrieval group resulted in any

significant differences in freezing during the first 4 trials of extinction (ps >.05) suggesting that the differences observed during fear acquisition are a result of differential responses to the immediate presence of the foot-shock as opposed to differences in the ability to acquire and retain CS-US association. Freezing during the extinction session was initially analyzed with a 2x2x2 mixed factor ANOVA with extinction cue as the repeated measure and retrieval group (ext only, ret+ext), orienting classification (Orienters or Nonorienters), and shock intensity (0.7mA or 1.0mA) as the between subjects factors. Rats did show a significant reduction in freezing over the course of extinction as evidenced by a significant within-subjects effect of extinction cue, $F(18,1026)=62.53$, $p<0.001$, with no overall main effect of either orienting classification, $F(1,57)=0.05$, $p=0.831$, or retrieval group, $F(1,57)=2.40$, $p=0.127$.

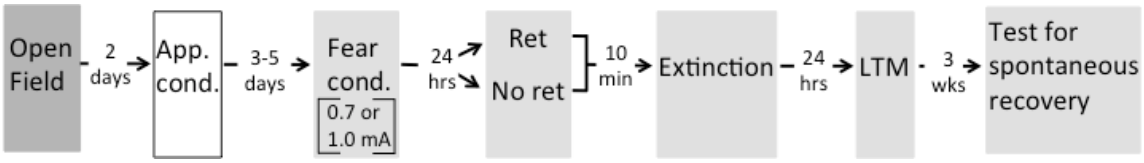
3.4.6. Long term memory of fear

Twenty-four hours after extinction, rats were tested for long-term memory (LTM) by presenting 4 tone-only trials (variable ITI=120s) in the same context as fear conditioning and extinction. Freezing behavior during these trials was scored and averaged During the LTM test, none of the experimental groups showed a significant increase in freezing, as compared to their own freezing at the end of extinction (all p's > 0.1). For rats conditioned with a 0.7 mA shock, no between-group differences existed in LTM freezing. For rats conditioned with a 1.0 mA shock, the freezing levels of Orienters and Nonorienters receiving typical extinction treatment (ext only) were comparable; however, Nonorienters in the ret+ext group showed significantly higher freezing than Orienters in the ret+ext group, $t(15) = 2.89$, $p=0.011$.

3.4.7. Spontaneous recovery of fear

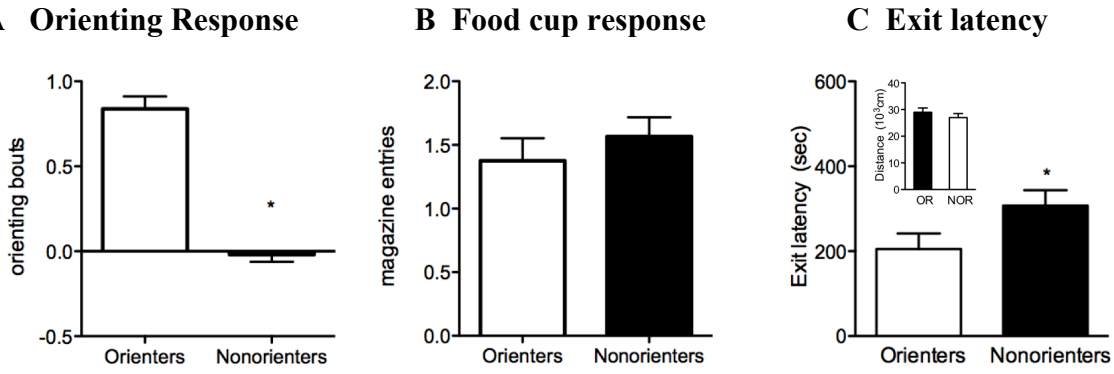
Twenty-one days after extinction, rats were returned to the chambers and tested for spontaneous recovery (SR) of freezing by playing 4 tone-only trials (variable ITI=120s). An overall ANOVA with orienting classification, shock intensity, and retrieval group as the factors revealed no overall effect of orienting classification, $F(1,57)=0.19$; $p=0.661$, but did reveal a significant overall effect of both retrieval group, $F(1,57)=10.02$; $p=0.002$, and shock intensity, $F(1,57)=16.05$; $p<0.001$, as well as a significant orienting classification X shock intensity interaction, $F(1,57)=5.75$; $p=0.02$, and a trend towards an orienting classification X shock intensity X retrieval group interaction, $F(1,57)=3.73$; $p=0.058$ (Fig 4). Rats receiving typical extinction treatment (ext only) showed recovery of freezing, regardless of orienting classification or shock intensity, i.e. freezing was significantly increased from extinction to the SR test [Orienters – 0.7 mA: $t(8) = 3.133$, $p = 0.014$; Nonorienters – 0.7 mA: $t(5) = 2.96$, $p = 0.032$; Orienters – 1.0 mA: $t(8) = 7.73$, $p < 0.001$; Nonorienters – 1.0 mA: $t(6) = 2.785$, $p = 0.032$]. In contrast, rats exposed to a retrieval trial prior to extinction did not show significant recovery of freezing during the SR test regardless of orienting classification or shock intensity (all p's > 0.2). Although neither Orienters nor Nonorienters receiving a retrieval trial prior to extinction showed a significant increase in freezing from extinction to SR test, for either shock intensity, Nonorienters showed more freezing behavior during SR test than Orienters after conditioning to a 1.0mA shock, $t(15) = 2.18$, $p=.045$, and less freezing behavior than Orienters after conditioning to a 0.7mA shock, $t(15)=2.23$, $p=0.041$.

Figure 3.1. Timeline of experimental design



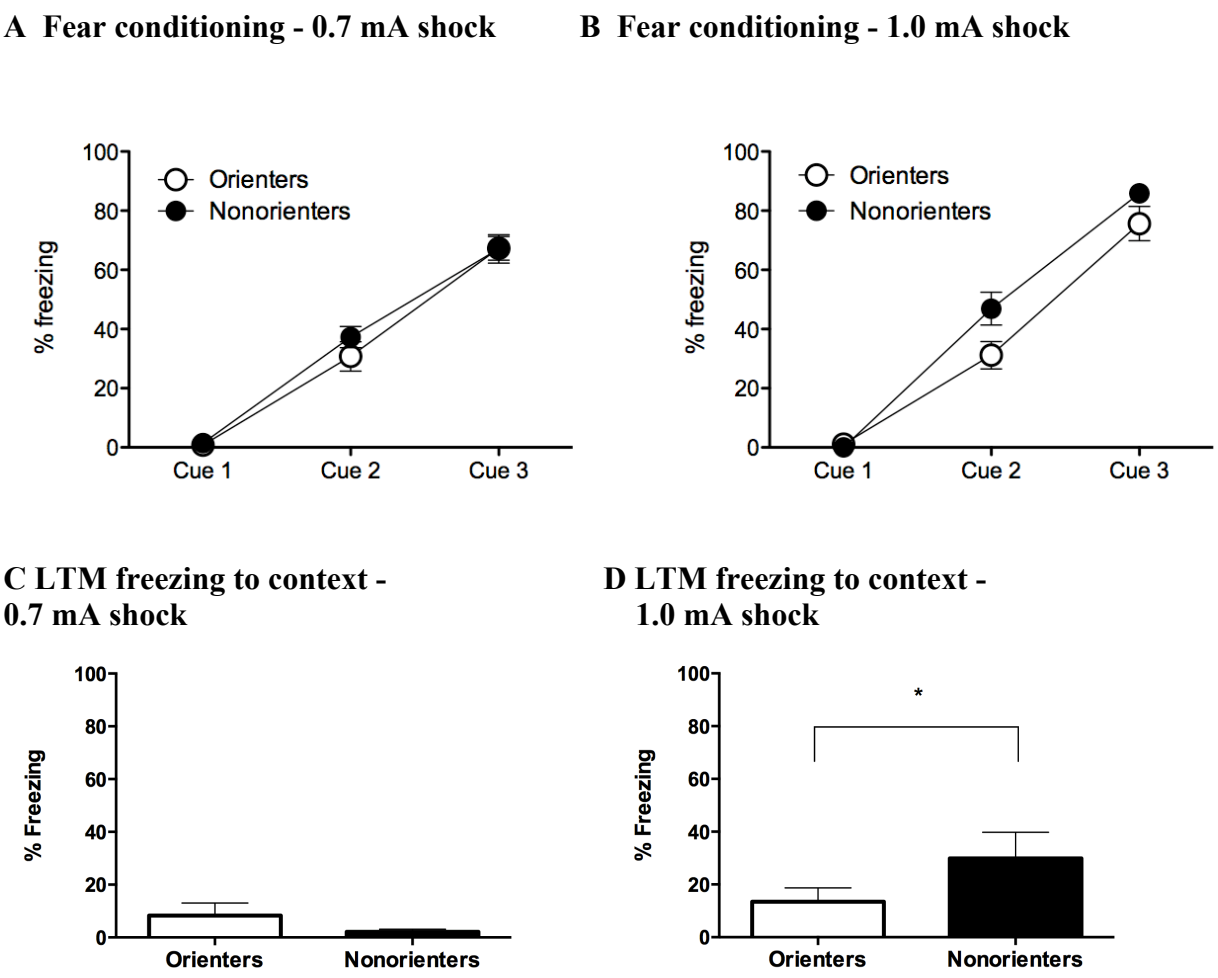
Rats were first tested for their willingness to enter an illuminated open field. Rats then received appetitive conditioning (App. cond.) with 56 light-food pairings in Context A. On their last day of appetitive conditioning rats were classified as Orienters and Nonorienters. After 3 to 5 days, both groups were fear conditioned (Fear cond.) with 3 tone-shock pairings of either 0.7 or 1.0 mA in Context B (indicated by grey shading). 24 hours after fear conditioning, rats were exposed to a single cue retrieval trial (Ret) or a typical extinction session (No ret). For rats in the Ret group that received a cue exposure and those in the No ret group that received a context exposure, the exposure occurred 10 minutes prior to beginning the extinction session. 24 hours after extinction, rats were tested for long-term memory (LTM), and 3 weeks later tested for spontaneous recovery. Context change is indicated by shading.

Figure 3.2. Acquistion of orienting and food cup responses, dark box exit latency



(A&B) Conditioned orienting and food cup approach. Mean ± SEM number of orienting bouts (*p<0.0001) (B) or food cup entries (p=0.42) (C) averaged for last 8 trials of training. Orienters showed significantly more OR than Nonorienters, but food cup response was equivalent between groups. (C) Latency to exit the dark insert and enter the illuminated open field. Orienters exited more quickly (p=0.05). Activity, as measured by the total distance traveled within both fields (insert), did not differ between Orienters and Nonorienters (p=0.37).

Figure 3.3. Freezing to tone and context after conditioning with 0.7 and 1.0 mA shock

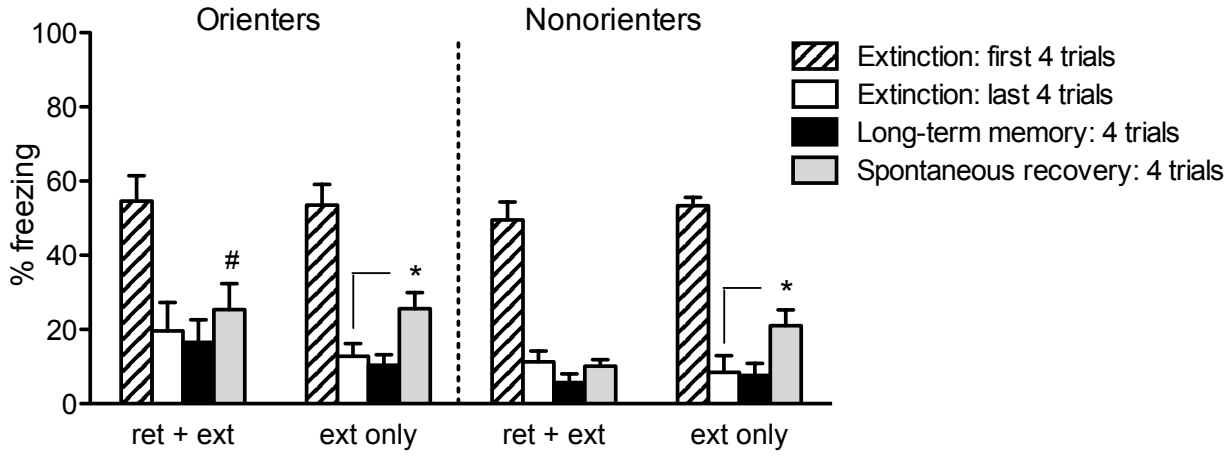


(A&B) Freezing during fear conditioning with a 0.7mA and 1.0mA footshock. (A) Orienters and Non-orienters showed no differences in freezing during conditioning when the US was a 0.7mA footshock (p=.49). (B) Non-orienters froze significantly more than Orienters during fear conditioning when the US was 1.0mA footshock (p=.04). Each conditioning session involved three CS-US pairings. (C&D) Contextual freezing 24 hours after fear conditioning to either a 0.7mA or 1.0mA footshock. (C) There were no significant differences between Orienters and Non-orienters in freezing to the fear

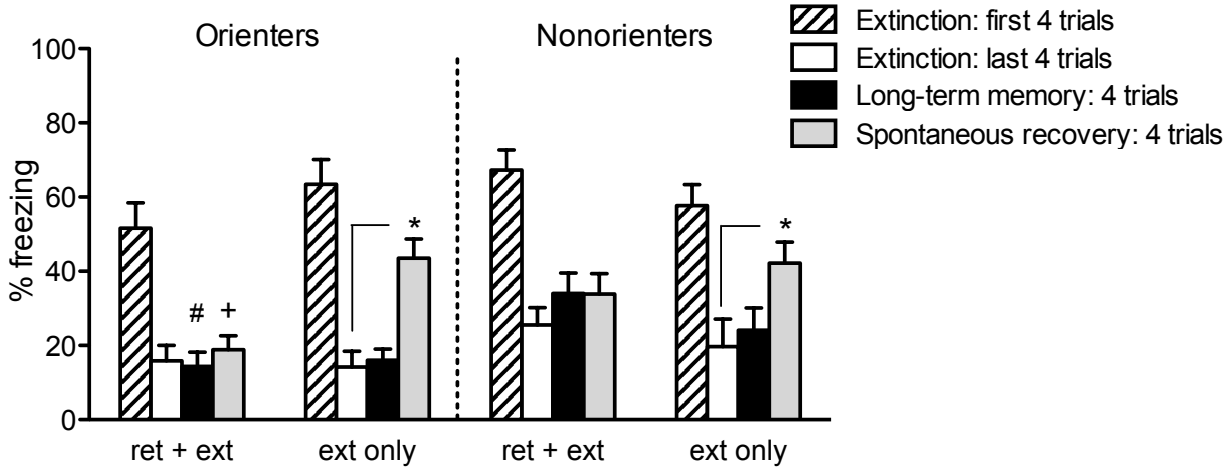
conditioning context when the US was 0.7mA footshock (p=.09) and overall context freezing was extremely low. (D) Non-orienters froze significantly more than Orienters to the fear conditioning context when the US was a 1.0mA footshock (*p=.03).

Figure 3.4. Cue-induced freezing at the beginning of extinction, end of extinction, during LTM test, and spontaneous recovery

A 0.7 mA



B 1.0 mA



(A) For rats conditioned with a 0.7 mA shock, a retrieval trial prevented spontaneous recovery (i.e. there was no significant increase in freezing from the end of extinction to spontaneous recovery test; Orienters p=0.206, Nonorienters p = 0.732). While neither group showed significant spontaneous recovery, Nonorienters froze significantly less than Orienters during the test for spontaneous recovery ($^{\#}p = 0.041$). Rats receiving typical extinction treatment did show a significant increase in freezing (Orienters p = 0.014, Nonorienters p = 0.032). (B) Rats conditioned with a 1.0 mA shock showed the same pattern of results: a retrieval trial prior to extinction attenuated spontaneous recovery (Orienters p = 0.524, Nonorienters p = 0.235). Rats exposed to typical extinction showed a significant increase in freezing (Orienters $p < 0.001$, Nonorienters p = 0.032). While neither Orienters nor Nonorienters that received ret+ext showed significant increases in freezing from the end extinction to LTM or spontaneous recovery tests, Orienters showed significantly less freezing than Nonorienters at both time points (LTM $^{\#}p = 0.011$, spontaneous recovery $^{+}p = 0.045$).

3.5. DISCUSSION

Fear conditioning provides a controlled means to investigate aversive associations that underlie many pathological fear conditions. Memory update methods such as ret+ext, where an extinction session is presented within the reconsolidation window show promise for reducing fear non-invasively; however, individual differences between subjects and methodological variations across laboratories leaves the efficacy of such paradigms in question. Here we consider how individual differences in response style during an appetitive conditioning task (i.e. propensity for conditioned orienting to a light stimulus predictive of food) relate to individuals' hesitance to enter an open field and how they affect freezing after fear conditioning. We report that Nonorienters show more reluctance to enter an illuminated open field, indicating an enhanced fear of unfamiliar open environments, as compared to Orienters. Additionally, we report that when conditioned with a tone and 1.0 mA footshock, Nonorienters show heightened freezing. Groups do not differ in their response conditioning with a 0.7 mA shock.

After fear conditioning to a foot-shock of either standard intensity (0.7 mA) or increased intensity (1.0 mA), ret+ext prevented spontaneous recovery of freezing for both Orienters and Nonorienters. However, Nonorienters in the ret+ext group froze significantly more than Orienters in the ret+ext group. We show that while retrieval+extinction prevents the significant return of fear for both phenotypes, the intensity of the US used in training and subjects' appetitive phenotype affect the magnitude of fear behavior that persists. A relationship between these two behaviors (conditioned orienting in an appetitive task and fear expression in a fear conditioning task) seems perhaps unsurprising given the overlap in the neural circuitry responsible for each. Projections from the central nucleus of the amygdala have been shown to be necessary for both the acquisition of conditioned orienting to a cue predictive of reward

and the freezing response exhibited after fear conditioning (LeDoux et al., 1988; Gallagher et al., 1990; Han et al., 1997; Goosens and Maren, 2001; Choi and Brown, 2003; Duvarci et al., 2011). It is possible Orienters and Nonorienters have fundamental differences in central amygdala function and that the results reported here are evidence of that variation, but more investigation needs to be done.

Furthermore, we report that after conditioning to a strong 1.0mA footshock, Nonorienters show increased susceptibility to condition to context than Orienters as evidenced by increased freezing in the absence of the CS when returned to the chamber 24 hours after conditioning. This result replicates previous research indicating that goal-trackers show more context-induced freezing when placed in the conditioning context 24 hours after aversive conditioning (Morrow et al., 2011). However, the same study also reported that sign-trackers show more cue-induced freezing when first re-exposed to an aversive CS, while we report that the two groups show no difference when initially re-exposed to the tone. Morrow et al., (2011) reports freezing results during re-exposure to the CS in a novel context, 24 hours after conditioning, while we report freezing during CS exposure in the original conditioning context both 24 hours after conditioning and 21 days after extinction or retrieval+extinction. Another difference lies in characterization of sign-tracking phenotypes. Morrow et al., used insertion of an inactive lever as a CS which elicited a different form of sign-tracking behavior (i.e., engagement with the lever). Unlike Orienters that also displayed US-directed food-cup behavior, these rats engaged almost exclusively with the lever while others engaged almost exclusively with the food cup resulting in an inverse correlation between these two behaviors. These two types of sign-tracking behaviors (i.e., lever-engagement and orienting) might represent slightly different phenotypes. It has been shown that the central nucleus of the amygdala,

which is crucial for acquisition of conditioned orienting (Gallagher et al., 1990), is not necessary for sign-tracking behavior towards the lever CS (Chang et al., 2012).

Nonorienters' apprehension about entering an open field, enhanced freezing during fear conditioning, and enhanced expression of contextual fear suggest that their expression of fearful behaviors differs from that of Orienters across modalities and circumstances. Although retrieval+extinction prevents spontaneous recovery in all cases, conditioning to a 1.0 mA footshock resulted in Nonorienters freezing more than Orienters during tests both 24 hours (LTM) and 21 days (SR) after retrieval+extinction, whereas conditioning to a 0.7 mA foot shock resulted in Orienters freezing more than Nonorienters during a test 21 days after retrieval+extinction. These differences in freezing after conditioning to a 0.7 mA foot shock were not present 24 hours after retrieval+extinction. Combined, our results suggest that time, orienting phenotype, and shock intensity all interact to influence the ability of an extinction session within the reconsolidation window to update an existing fear memory trace. The influence of these factors on the efficacy of retrieval+extinction may provide some explanation for the variation in reported results for fear memory updating studies. Understanding individual differences and their neurobiological correlates is key to understanding memory update techniques.

Chapter 4: Impulsivity, risk-taking, and distractibility in rats exhibiting robust conditioned orienting behaviors

4.1. ABSTRACT

When a neutral cue is followed by a significant event such as food delivery, some animals become engaged with the cue itself and acquire cue-directed behaviors. These animals seem to show behavioral impulsivity and altered attention, as compared to those rats showing behavior directed toward the reward delivery location. The vast majority of the work characterizing the phenotype of rats showing robust cue-directed behavior is based on a particular procedure in which insertion of a lever is used as a conditioned stimulus (CS) and the animal's contact to/interaction with the lever is used as a measure of cue-approach. The current study used a light CS to categorize rats' propensity for cue-directed responding, and assessed whether individual differences in impulsivity and related behaviors (such as risk taking and inattention) still emerged. During the light CS and food pairings, some rats displayed enhanced rearing/orienting at the onset of the light CS (Orienters) prior to showing food-cup approach behavior, while other rats only showed food-cup approach behavior (Nonorienters). We examined whether Orienters and Nonorienters differed in their performance of a variety of tasks used to measure different aspects of impulsive and risky behaviors, as well as attentional function. The results showed that Orienters made more impulsive and risky choices, and were quicker than Nonorienters to leave a familiar dark environment to enter a novel open bright field. When tested in a 5-choice serial reaction time task, Orienters showed less accuracy in target detection when a visual distractor was introduced during an attentional challenge. Our current study suggests that light CS-induced rearing/orienting behavior might not

necessarily share similar mechanisms with lever CS-approach behavior in predicting impulsivity-related behaviors.

4.2. INTRODUCTION

After repeated pairings of an environmental cue (conditioned stimulus, CS) and a biologically significant event such as food delivery (unconditioned stimulus, US), animals acquire various conditioned responses (CRs). One prominent observable CR is directed toward the reward delivery site in anticipation of receiving the reward at the end of the CS presentation. However, some animals display CRs that are directed toward the CS itself. Individual variation in the frequency of these behaviors has been widely reported across species including pigeons (Balsam et al., 1978), mice (Tomie et al., 2012) and rats (Locurto, 1981), such that some subjects show reliably high levels of such behaviors, whereas others do not. As these behaviors are thought to reflect the incentive salience attributed to the cue (that is, conditioned attentional, emotional, and/or motivational properties acquired by the cue), they suggest a different nature of cue information processing in subjects with high vs. low levels of these conditioned behaviors (Cardinal et al., 2012; Holland, 1977; Robbins and Everitt, 1996; Robinson and Berridge, 2007; Olshavsky et al., 2013a,b).

One common form of these behaviors is sign-tracking (or autoshaping), and consists of approach to and contact with a cue (such as insertion of a lever into the test chamber) predictive of reward delivery. Importantly, even though sign-tracking behaviors are not required to obtain the reward (and may in fact interfere with obtaining the reward), animals continue to display these behaviors, which are often difficult to suppress and tend to recover quickly after extinction (Barrera, 1974; Davey et al., 1981; Kearns and Weiss, 2007; Schwartz and Williams, 1972). As a consequence, sign-tracking

behaviors have been proposed to reflect a form of impulsivity or reduced inhibitory control (Tomie et al., 2008). However, very few studies have directly addressed the relationship between sign-tracking and impulsive behaviors (Flagel et al., 2010; Lovic et al., 2011). Impulsivity can manifest in several forms and is associated with various disorders, such as drug addiction and ADHD (Olmstead, 2006; Dalley and Roiser, 2012). The symptoms of pathological impulsivity in humans may be classified as a lack of motor inhibition, lack of attention/vigilance, or preference for small immediate rewards over larger delayed rewards (Robbins et al., 2012). Typically, impulsivity is divided into two major categories: impulsive choice (also cognitive impulsivity) and impulsive action (also motor impulsivity) (Dalley et al., 2011; Evenden, 1999). Impulsive choice is evident when an individual selects a smaller immediate reward over a larger delayed reward. Impulsive actions refer to those behaviors that occur due to a failure of inhibitory control such as premature or poorly-timed responding. Studies by Flagel et al. (2010) and Lovic et al. (2011) reported that rats with high levels of sign-tracking also displayed greater impulsive action, but less impulsive choice.

Here we describe a form of cue-directed behavior (i.e., conditioned orienting) different from that characterized in the previously mentioned sign-tracking studies. Then, we examine the relationship between conditioned orienting and impulsivity as well as related behaviors (risk taking and inattention). Conditioned orienting is evident in Pavlovian conditioning tasks in which a light CS is paired with a food pellet reward. Rats develop two readily observable CRs to the light CS: approaching the site of food delivery (a reward-directed CR) and orienting/rearing in response to the light CS (a CR not directed toward the reward site). Conditioned orienting typically occurs within the first 5 seconds of a 10 second CS, whereas food cup approach occurs more frequently within the last 5 seconds of the CS directly preceding delivery of the reward (Holland, 1977). As

such, rats can display both behaviors within a single CS presentation and performance of orienting responses (ORs) does not preclude food cup approach. In our laboratory, some rats show robust light CS-elicited ORs (Orienters) whereas others do not (Nonorienters). However, these groups display comparable levels of US-directed food cup approach behavior as well as unconditioned spontaneous orienting (Olshavsky et al., 2013a,b).

In the current study, we tested Orienters and Nonorienters in an array of behavioral tasks to characterize impulsivity and related traits, and to determine the extent to which the Orienting/Nonorienting phenotype is predictive of this spectrum of behaviors. Specifically, rats were tested for impulsive choice using a delay discounting task, and risky decision-making behavior and novelty preference, both of which may be related to impulsive traits (Evenden, 1999; Molander et al., 2011). Then, another impulsive trait, inattention, was assessed using a five-choice serial reaction time task (5CSRTT).

4.3. METHODS

4.3.1. Subjects

A total of 55 male Long-Evans rats (Charles River) weighing 250-275 g upon arrival to the colony were used for these studies. Rats were housed in a reversed 14 h light/10 h dark cycle, with the lights off at 10 am. One week after arrival, rats were food restricted to maintain 90% of their free-feeding body weight; this weight was maintained throughout experiment 1 (n=25). For experiment 2 (n=14), food restriction to 90% free-feeding weight was maintained only for initial Pavlovian conditioning, and food was available *ad libitum* while they completed the novel open field task. For experiment 3 (n=16), one week after arrival, rats were food restricted to maintain 90% of their free-

feeding body weight; this weight was maintained as in experiment 1. All experiments were conducted according to *the National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

4.3.2. Apparatus

Initial light-food pairings took place in a conditioning chamber with aluminum sidewalls and ceiling, and clear acrylic front and back walls (30.5 W x 25.5 D x 30.5 H in cm, Coulbourn Instruments, Whitehall, PA). The floor was made of stainless steel rods (0.5 cm in diameter, spaced 1.0 cm apart). The food cup was located on the right-hand wall of the chamber, 2.5 cm above the floor. Nosepoke entry into the food cup was detected by an infrared beam at the opening. A 2-Watt white light was mounted 20 cm above the food cup and its illumination served as a CS signaling grain pellet delivery (45 mg TestDiet, Richmond, IN). The wall opposite the food cup was concave and had five ports which remained inactive during the Pavlovian conditioning procedures. Each chamber was enclosed in a light- and sound-attenuating box (58.4 cm x 61 cm x 46 cm) in which the ventilation fan provided masking noise. A video camera was mounted within each box and images were recorded during behavioral training. The 5CSRTT also took place in this conditioning chamber. During this task, the five ports opposite the food cup were active and nosepokes to the ports were detected by infrared beams at the openings.

The delay discounting and risky decision-making tasks were carried out in conditioning chambers that were different in size and shape (Coulbourn). The chambers had aluminum side walls and ceiling, and acrylic front and back walls (25.4 W x 25.4 D x 29.2 H in cm). A food cup, into which grain pellets were delivered, was located 2.5 cm

above the floor, and a 1-Watt house light outside of the chamber (i.e., mounted on the wall of the sound- and light attenuating box) was used to signal the beginning of each trial. The house light bulb, not visible to the rats, provided diffuse and dim illumination to the chamber. Retractable levers were placed to the left and right of the food cup. The wall opposite the cup and levers had a retractable door that remained closed during these tasks.

The assay for novelty preference took place in an open field chamber consisting of a white acrylic floor surrounded on all sides by clear acrylic walls (43.2 W x 43.2 D x 30.5 H in cm). After testing the animals in the open field for general activity level, a black acrylic insert was placed into the open field. The insert was a rectangular prism with no floor, constructed of four side walls and a top (43.3 W x 21.6 D x 30.5 H in cm) so that the white floor of the open field remained exposed. The side facing the rest of the open field had a small opening to allow the rats to freely move in and out of the insert. Activity was detected by arrays of infrared light beam motion detectors (16 x 16, 2.5 cm apart) at the sides of the chamber, thus creating a detection grid. Two arrays of detectors were located 1 cm and 13 cm above the floor to monitor horizontal and vertical activity. Activity Monitor program, version 5.10 (Med Associates, St. Albans, VT) was used to control the chamber and collect the data.

4.3.3. Procedures: Experiment 1

4.3.3.1. Light-food pairings

Twenty-five rats were trained on a simple appetitive Pavlovian conditioning procedure in order to classify them as Orienters and Nonorienters. First, rats underwent a pre-training session in which they were trained to eat a single grain pellet delivered to a

food cup located within the conditioning chamber. A total of 30 pellets were delivered at a variable interval (averaging 60 s) over a 30-min session. After this session, all rats reliably retrieved grain pellets from the cup. The first training session consisted of two parts. In order to habituate the unconditioned orienting response to light, the stimulus light was illuminated eight times, for 10 s each time, without any food pellets being delivered to the cup. Then, during the second half of the session, 8 trials of a 10 s light presentation were followed by a food pellet delivery to the food cup. For the next three days of conditioning, sessions consisted of 16 light – food pairings with a variable intertrial interval (ITI) averaging 120 +/- 60 seconds.

ORs were scored from DVD recordings of all training sessions. During the 5 s prior to the CS and the 10-s light presentation, OR was sampled every 1.25 s. At each time point, the presence of OR was recorded allowing for 12 observation points per trial. An OR was defined as a rearing response in which both forelimbs were lifted from the floor of the conditioning box, and did not include grooming behavior. Because the chamber was completely dark during the ITI and the onset of the light provided diffuse illumination of the entire conditioning chamber, rearing in a direction other than that of the light bulb was also considered an OR. Therefore, OR doesn't exclusively represent rearing directly towards the light CS, but includes any rearing behavior. In the original report and detailed analyses of conditioned orienting behavior (Holland, 1977), only half of what was considered conditioned orienting was rearing directed to the light source. Since then, all the existing work examining the behavioral and neural mechanisms of conditioned ORs has employed similar scoring methods to those described here (El-Amamy and Holland, 2006, 2007; Gallagher et al., 1990; Han et al., 1997; Holland, 2007; Holland and Gallagher, 1999; Lee et al., 2005, 2010, 2011; McDannald et al., 2004). Food cup approach is reported as counts of nosepokes into the magazine as measured by

the infrared beam. Like ORs, food cup approach was measured during the 5 s prior to the CS and during the 10-s light presentation.

When presented with a 10-s light CS that predicts pellet delivery into a magazine, rats typically display ORs during the first five seconds (first half) and a food cup approach response during the last five seconds (second half). Numerous studies using different CSs, procedures, and species, have reported this phenomenon (Buzsaki, 1982, Holland, 1977, Lee et al., 2011). Thus, ORs from the first half of the light presentation (CS1) and food cup approach from the second half of the light presentation (CS2) will be considered in examining individual differences in the acquisition of OR and food cup approach. To account for individual variation in baseline behavior, we report the difference between the responses during CS1 or CS2 presentation and pre-CS responding during the 5 s prior to the CS onset. For example, a rat displaying an OR at 1 observation point during the pre-CS period and 3 observation points during CS1 would receive an OR score of 2 on that trial. For the same reason, a rat with a food cup approach score of 1 during the pre CS period and a 3 during CS2 would receive a food cup approach score of 2 on that trial.

4.3.3.2. Delay discounting

Following completion of appetitive Pavlovian conditioning, the rats were trained in the delay discounting task. First, rats were shaped to press a single lever (either left or right – counterbalanced across groups), while the second lever was retracted. After reaching a criterion of 50 presses during a single 30-minute session, they were shaped to press the opposite lever. After reaching criterion on the opposite lever, rats were shaped to nosepoke to the food magazine upon its simultaneous illumination with the house light. Nosepoke to the magazine resulted in the extension of a single lever, either left or right.

A press to the lever caused the delivery of a grain pellet to the magazine, as well as the extinguishing of the magazine and house lights and retraction of the lever. Each lever was presented an equal number of times within the 60-min session (ITI 40±10s), with no lever being presented more than twice consecutively. Once rats reached a criterion of 60 lever presses per session, they began the delay discounting task.

Each session of the delay discounting task was comprised of 5 blocks of 12 60-s trials. Each trial began with a 10-s illumination of the magazine and house lights. Nosepoke to the magazine resulted in the extension of either a single lever (forced choice trials) or both levers (free choice trials). For each block of trials, the first 2 trials were forced choice trials and the remaining 10 trials were free choice trials. Presses to one lever (either left or right, counterbalanced across groups) resulted in the delivery of one pellet to the magazine, while presses to the opposite lever resulted in the delivery of three pellets after varying delays. Once a lever was pressed, the levers were retracted and the house light was extinguished until the time of food pellet delivery. Food delivery resulted in the re-illumination of both magazine and house lights. The lights were again extinguished upon nose-poke to the magazine or after 10 s, whichever occurred first. Trials in which rats failed to nosepoke were scored as omissions. During the first block of 12 trials, the delay was 0 s. In the following blocks, delay increased to 4, 8, 16, and 32 s. Because trial durations were fixed, choice of the small reward did not result in sooner opportunities for subsequent rewards. Objectively, the large delayed reward was the most favorable choice as it resulted in more food delivery over the course of each session. We report the percentage of trials in which rats chose the larger reward (i.e. number of trials choosing larger reward/number of completed trials). Rats received daily training sessions for 25 days, at which point responding was stable. Stable responding was defined by the absence of a main effect or interaction involving the last 5 days (days 21-

25) in a repeated measures ANOVA (day x delay), accompanied by a significant main effect of delay.

The design of the delay discounting task (ascending fixed delay durations tested within each session) was based on that developed by Evenden & Ryan (1999) and Cardinal et al. (2001). This task design has been used extensively to investigate the pharmacological and neural basis of delay discounting (e.g., see review paper, Floresco et al., 2008). A number of other task designs are possible; however, the design employed here offers several advantages. First, the within-session changes in delay allow testing of multiple delay durations within a single session, which provides for more rapid assessment of performance across a range of delay conditions. Second, because the ascending delay design has been used extensively, it provides considerable comparative power with the previous work. Finally, it should be noted that we have shown previously using the within-session fixed delay design and ascending and descending series of delays produce comparable outcomes (Simon et al. 2010).

4.3.3.3. Risky decision-making

Upon completion of the delay discounting task, the same rats began testing in the risky decision-making task as previously described in Simon et al. (2009). This task was similar to the delay discounting task in that 60-min sessions were comprised of five blocks of trials and that each trial began with a 10-s illumination of the magazine and house light. Again, presses to one lever resulted in the delivery of a small reward, while presses to the opposite lever resulted in the delivery of a larger reward. However, during the risky decision-making task, trial blocks consisted of 18 trials: 8 forced choice trials and 10 free choice trials. Also, the small reward was maintained at one pellet, while the large reward was increased to four pellets. In addition, choosing the large reward

resulted not in delay to reward, but in the risk of receiving a 500 ms 0.35 mA footshock. During the first block of trials, the probability of shock was 0%, and increased to 25, 50, 75, and 100% across subsequent blocks (note that the large food reward was delivered after every choice of the large reward lever, irrespective of shock delivery). Rats were tested on the risky decision making task for 15 days, until responding was stable across the final five days (11-15). Again, stable responding was defined by the absence of a main effect or interaction involving days in a repeated measures ANOVA (day x shock probability), accompanied by a significant main effect of shock probability.

In order to confirm that Orienters and Nonorienters did not differ in their unconditioned response to shock, a shock sensitivity test was conducted following the risky decision-making task (Kim et al., 1991; Kosten et al., 2006). In this test, rats were given repeated footshocks at 10 s intervals. At the beginning of the session, shock was 0.1 mA and increased by 0.05 mA every trial. An experimenter blind to rats' orienting categorization recorded the shock amplitude at which rats flinched and the amplitude at which they jumped.

4.3.3.4. Five-choice task

After completing both the delay discounting and risky decision making tasks, 16 of the 25 rats used above were then trained on a 5-choice serial reaction time task (5CSRTT); this cohort included 9 Orienters and 7 Nonorienters. Daily training sessions were approximately 40 minutes and consisted of 60 trials. In this task, a 5-s house light illumination served as a ready signal for the illumination of one of five ports. A nosepoke to the illuminated port resulted in the delivery of a food pellet to a food cup located on the opposite wall of the chamber. Nosepokes prior to port illumination or to the incorrect port were recorded but not punished. Rats were initially shaped to respond to a 30-s port

light illumination. A nosepoke to the correct port at any time during the 30 seconds resulted in food pellet delivery and the extinguishing of the house light. Once rats correctly responded to 80% of trials in a session, the port light duration was gradually shortened for the following session to eventually reach 500 ms (i.e., 30, 20, 15, 10, 5, 3, 1, 0.5 s). Training continued until rats successfully completed two out of three consecutive sessions with a 500 ms port illumination. Once rats reached this criterion, they received three attentional challenges. These tests included shortening the port light duration from 500 to 100 ms, varying the duration of the ready signal to 1, 5, or 9 s from fixed 5 s, and blinking of the house light ready signal instead of a steady signal (Robbins, 2002). Attentional challenge test sessions were separated by 500 ms port light sessions, such that three test sessions and two 500 ms sessions were completed over five consecutive days. During the sessions consisting of port illumination durations of less than 5 s, rats had a total of 5 s from the port illumination to make a nose-poke to receive the food pellet. For example, during the 500-ms sessions, there was a 4.5-s limited hold period for nosepoke after port illumination. In order to assess performance, we consider both the percentage of correct trials and the number of premature responses (i.e. nosepokes to the five ports during the ready period).

4.3.4. Procedures: Experiment 2

4.3.4.1. Light-food pairings

The rats (n=14) used for the open field task were from another study in which they underwent Pavlovian conditioning identical to the one described in Experiment 1. In addition, they also experienced one session of 18 extinction trials (i.e., 18 light CS alone

presentations) 24-hrs after their last training session and, 3 weeks later, a session consisting of 4 light CS alone presentations to test for spontaneous recovery of CRs.

4.3.4.2. Open field and light-dark emergence tasks

After the completion of appetitive training, extinction, and test for spontaneous recovery, the rats completed an open field task that took place over 4 consecutive days. On the first 2 days, they were placed in the open field for 10 min to measure activity levels when the environment was novel (day 1) and when it was familiar (day 2). During the last two days, the open field was modified by inserting a dark box that covered half of the activity chamber and affixing checkerboard-patterned papers to the outside walls to create a novel environment outside the dark box. On the third day, rats were confined to the dark compartment for 10 minutes. On the fourth day, rats were initially placed in the dark compartment, but were free to exit into the illuminated (100 lux) novel compartment. They were allowed free access to either side for 10 minutes. Time and distance traveled within the dark compartment and novel field provided measures of novel place preference. Latency to exit the dark compartment provided measures of impulsivity. The sensors on the apparatus' floor calculated a point for the rat's center of gravity, which equated to about the rat's midsection. A human observer blind to the rat's groups confirmed the computer measures of exit latency by recording the time when the rat's front paws, head, and shoulders were outside of the dark box prior to completely exiting the box.

4.3.5 Procedures: Experiment 3

The rats used for this task (n=16) were experimentally naïve and underwent food cup training, habituation, and light-food pairings identical to the procedures described in experiment 1. Following Pavlovian training procedures all rats underwent behavioral

training similar to the risky decision-making procedures described above (3.3.3.3). One important difference existed. For experiment 3 presses to either lever resulted in the delivery of a single grain pellet. Rats were trained in this procedure until responding was stable across the final five days (days 19-23).

4.3.6. Data analyses

For the appetitive acquisition data in experiment 1, we conducted an orienting classification (Orienters vs. Nonorienters) x two-way repeated ANOVA, trial block (7 blocks of 8 trials each) and CS time (CS1 and CS2). For both the delay discounting and risky decision-making tasks, orienting classification x two-way repeated ANOVAs (last 5 training sessions x 5 delay or shock sessions) were conducted. Furthermore, linear regression analyses were conducted between orienting scores and delay discounting scores and risky decision-making scores. For the 5CSRTT, an orienting classification x attentional challenge (baseline, 100ms, variable ready period, blinking light) repeated measures ANOVA was performed. A significant main or interaction effect was followed by post-hoc Bonferroni tests. In addition, linear regression analyses were also conducted between orienting scores and the accuracy (i.e., percent trials with the correct response).

For the appetitive acquisition data in experiment 2, we conducted an orienting classification x trial block repeated ANOVA for OR during CS1 and food cup during CS2. A significant interaction effect was followed by a t-test on the last trial block. For the open field test, orienting classification x test day repeated ANOVA was conducted on the first 2 days of regular open field tests. For the dark-light emergence test on day 4, t-tests were conducted between Orienters' and Nonorienters' activity levels and distance traveled either in the dark box or in the bright open area. For the exit latency of the dark

area, log-rank test was used to determine how quickly each group emerged to the bright area.

Appetitive acquisition data from experiment 3 were analyzed as in experiment 2, with an orienting classification x trial block repeated ANOVA for OR during CS1 and food cup during CS2. Risky decision-making data were analyzed as in experiment 1, with an orienting classification x two-way repeated ANOVAs (last 5 training sessions x 5 shock sessions).

4.4. RESULTS

4.4.1. Experiment 1

4.4.1.1. Light-food pairings

Over the course of training, rats acquired significantly more conditioned OR during the first half of the CS, as shown by an interaction effect of trials and CS time (i.e., CS1 and CS2), $F(6, 138)=3.71$, $p<0.01$ (Figure 4.1A left panel). However, a subset of rats did not display persistent conditioned OR. Thus, based on their average OR scores within CS1 during the last eight training trials, rats were divided into two groups (Figure 4.1A middle panel). Rats scoring above the median OR score were classified as Orienters (n= 13), while those rats that scored at or below the median score were classified as Nonorienters (n=12). Figure 4.1A right panel illustrates the acquisition data shown in the left panel re-organized based on the orienting classification. A significant interaction effect of trial and orienting classification, $F(6, 138) = 2.95$, $p < 0.05$, as well as a main effect of orienting classification, $F(1, 23) = 6.84$, $p < 0.05$, indicated that Orienters, but not Nonorienters, acquired robust OR behavior during training. The rats also received 8 light alone trials prior to light-food pairings to measure and habituate

unconditioned OR. There was no difference between Orienters and Nonorienters in their unconditioned orienting response, $F(1, 23)=0.6$, $p>0.4$. In addition, we also analyzed the probability of OR occurrence in a given trial over the course of training (i.e., score of 0 or 1 to indicate absence or presence of orienting in a single trial). Similar to the findings of OR counts, Orienters were significantly more likely to show OR than Nonorienters as shown by a main effect of orienting classification, $F(1, 23) = 10.6$, $p < 0.01$.

Regardless of OR level, all rats acquired a conditioned food cup response over the course of training as shown by the main effect of trials $F(6, 138) = 13.8$, $p < 0.001$. In accordance with earlier reports, the rats mainly exhibited conditioned food cup approach during the second half of CS presentation, CS2 (Fig 4.1B, left panel): there was a main effect of CS time, $F(1, 138) = 33.3$, $p<0.001$ and an interaction effect of trials and CS time, $F(6, 138) = 12.4$, $p<0.001$. Even though both Orienters and Nonorienters acquired conditioned food cup approach response, Nonorienters exhibited more food cup behavior than Orienters as shown by the main effect orienting classification, $F(1, 23) = 4.66$, $p<0.05$. However, the higher food cup response was only apparent during the second half of CS presentation as shown by three-way interaction of trials, orienting classification, and CS time, $F(6, 138) = 2.78$, $p < 0.05$. Again, further analyses on the probability of conditioned food cup response yielded comparable results: Nonorienters had more trials in which they exhibited conditioned food cup response, $F(1, 23) = 4.78$, $p<0.05$ and the probability was higher during the second half of CS presentation, $F(1, 138) = 4.865$, $p<0.001$. Figure 4.1B middle panel represents the individual food cup data scored during CS2 from the last 8 trials and shows similar food cup behavior between Orienters and a subset of Nonorienters while slightly higher food cup behavior is seen in other Nonorienters. Figure 4.1B right panel illustrates food cup acquisition data re-organized based on orienting classification.

4.4.1.2. Delay discounting

Each day, animals were given a 60-min session with 5 delay sessions of 0, 4, 8, 16, 32-s for the larger reward. After twenty-five days of training, all rats reached stable responding, as evidenced by no training day effect over the last 5 training days, $F(4, 92) = 0.81$, $p = 0.52$ and no interaction between day and delay, $F(16, 368) = 0.60$, $p = 0.67$). Figure 3.2A shows the average response of the last 5 training days (days 21-25). There was a significant main effect of delay, such that preference for the larger reward decreased as delay increased, $F(4,92) = 43.78$, $p < 0.001$ There was also a significant main effect of orienting classification, $F(1,23) = 4.13$, $p < 0.05$; there was no delay x orienting classification interaction, $F(4, 92) = 1.76$, $p = 0.14$. Orienters chose the immediate, but smaller, reward more as the delay for the larger reward increased. This resulted in Orienters receiving an average of 110 grain pellets, and Nonorienters receiving 126 pellets, $t(23) = 2.03$, $p = 0.054$, per session during the last 5 days of training. The marginally significant difference indicates that Orienters ultimately consumed fewer pellets by choosing the more immediate reward. Even though animals completed most trials throughout the session, both groups completed slightly fewer trials as delay increased (average over the last 5 sessions from 99.8% completion during no delay trials to 95% completion during 32-s delay trials), $F(4,92) = 11.30$, $p <0.01$. However, there was no main effect of orienting classification ($p = 0.64$) or an interaction effect ($p = 0.98$) for the trials completed. When considering the one- or three-pellet forced choice trials (administered at the beginning of each delay session block), rats averaged 98% completion overall. Neither the number of reinforced pellets nor the orienting classification made any difference in completion of the forced choice trials.

A regression analysis was also conducted to examine the relationship between orienting and delay discounting. In order to create discounting scores for each subject,

the AUC was calculated according to Myerson et al., 2001. Briefly, an equation for the trapezoid area, (x_2-x_1) $[(y_1+y_2)/2]$, was used, where x_1 and x_2 are successive delays and y_1 and y_2 are the subjective values associated with these delays. A regression analysis examining the influence of raw orienting score on delay discounting did not reveal a significant relationship; $R^2 = 0.11$, $F(1,23) = 2.83$, $p = 0.10$. However, because the probability density function of raw orienting scores was not normal (Figure 4.2C left panel), we normalized the orienting scores with a log transformation after adding a constant, 1.375 (Figure 4.2C right panel). Adding a constant was necessary to eliminate OR scores of zero in order to perform log transformation. The constant 1.375 was chosen so that the transformed OR score would start at zero since the lowest original OR score was -0.375. A regression analysis using the normalized orienting score revealed a marginally significant relationship, $R^2 = 0.15$, $F(1,23) = 3.92$, $p = 0.06$, in which rats with higher orienting scores tended to choose immediate small reward over larger reward as the delay for larger reward increased (Figure 4.2B).

4.4.1.3. Risky decision-making

After the completion of the delay discounting task, animals were trained on the risky decision-making task in which, instead of a delay period associated with the larger reward, there was an increasing chance of receiving a mild footshock associated with the larger reward. Animals reached stable responding after 15 days of training. Figure 3.3A shows the average response of the last 5 training days (days 11-15). As shock probability increased, both groups' preference for the larger reward decreased as evidenced by a significant main effect of shock probability, $F(4, 92) = 11.03$, $p < 0.001$. However, Orienters were more likely than Nonorienters to choose the larger reward. These findings

were supported by a significant main effect of orienting classification, $F(1,23) = 55.86$, $p < 0.05$.

Additionally, a regression plot with normalized orienting scores and the AUC scores revealed an almost bimodal distribution of the AUC scores and was unsuited for linear regression analysis (Fig 4.3B). With the exception of three individuals, the rats can be divided into high or low risk-taking groups (i.e., high or low AUCs, respectively). Division of high or low risk-takers based on orienting categorization revealed 9 high risk-taking and 2 low risk-taking choices among Orienters while 4 high risk-taking and 7 low risk-taking choices among Nonorienters. This observation suggests that Orienters are likely to make more risky decisions and choose larger rewards associated with potential footshock. A 2 (orienting classification) x2 (low vs high AUCs) Fisher's exact test approached marginal significance, $p=0.08$. Because of the non-linear nature of the AUC scores of the risky decision-making task, Spearman's non-parametric rank order correlation was conducted and showed that the OR scores significantly predicted the risky decision-making, $r=0.40$, $p<0.05$.

As a result of their preference for the larger reward, Orienters consumed significantly more pellets during choice trials than Nonorienters (164 and 107 pellets, respectively) during the last 5 days of training, $t(23) = 2.52$, $p < 0.05$. Even though animals completed most of the trials, as shock probability for the larger reward increased, rats in both groups completed slightly fewer trials: 99.7% completion for no shock to 97.5% completion for 100% shock. Orienters and Nonorienters did not differ in the number of trials they completed. This was confirmed by only a significant main effect of shock probability, $F(4,92) = 3.36$, $p < 0.05$, but no main effect or interaction involving orienting classification (p's >0.05).

A further regression analysis was conducted using the AUCs to examine whether performance during the delay discounting task predicted the performance during the risky decision-making task. In accordance with our previous finding (Simon et al., 2009), there was no relationship between these two tasks, $R^2 = 0.04$, $F(1,23) = 0.92$, $p > 0.3$.

Although rats ran no risk of receiving footshock when pressing the one-pellet lever, completion of the one-pellet forced choice trials was affected by increasing risk of shock on the four-pellet lever. Average percentage of forced choice trials completed decreased from 98.2% during the first trial block to 97.6% during the final trial block. An orienting classification x trial block repeated measure ANOVA of one-pellet forced choice trial completion showed a main effect of trial block, $F(4,92) = 3.01$, $p < 0.05$. There was no main effect of orienting classification ($p>0.7$) nor was there any orienting x trial block interaction ($p>0.9$). Rats also decreased completion rates of 4-pellet forced choice trials as risk of shock increased. On average, subjects completed 96.8% of 4 pellet force trials during the first trial block and only 77.2% during the final trial block. An orienting classification x trial block repeated ANOVA of four-pellet forced trial completion again revealed a main effect of trial block, $F(4,92) = 8.24$, $p<0.001$ and no main effect of orienting classification ($p>0.4$) nor interaction effect ($p>0.5$).

To confirm that differences of performance in the risky decision-making task were not due to inherent differences in shock sensitivity, the lowest shock amplitudes at which Orienters and Nonorienters flinched and jumped were measured (Figure 4.3C). Orienters and Nonorienters reacted equally to the increment of footshock intensity by flinching to the lower amplitude first and then jumping to the higher amplitude. There was neither a main effect of orienting classification ($p>0.3$) nor an interaction effect with shock increment ($p>0.8$). There was only a main effect of shock increment, $F(1, 23)=152.2$, $p<0.001$.

4.4.1.4. Five-choice task

Sixteen out of the initial 25 rats underwent the 5CSRTT. As evident in the orienting score distribution seen in the regression plots for 5CSRTT with 16 rats (Figure 4.4C) compared to the regression plots for earlier tasks using all 25 rats (Figure 4.2B and 4.3B), the ORs of the 16 rats were representative of the larger group. The average ORs from the last 8 trials were 0.92 and -0.02 for this subgroup of Orienters and Nonorienters, respectively, while the average scores of the larger group were 0.96 and -0.01.

When considering the pre-testing 500-ms port-light sessions, Orienters and Nonorienters did not differ in the mean number of sessions required to meet criterion of at least 80% correct trials, two out of three days in a row [Means: Orienters = 3; Nonorienters = 2.43, $t(14) = 0.956$, $p = 0.36$]. Analysis of performance during the 5CSRTT involved 2 components: a comparison of the number of nosepokes during the ready signal prior to port illumination (to measure premature responding) and a comparison of the percent trials with a correct nosepoke to the illuminated port (to measure attentional performance). During the baseline training with 500-ms port illumination, Orienters and Nonorienters performed comparably. On the last baseline training day with 500 ms port illumination (white bars in Figure 4.4), there was no significant difference between Orienters and Nonorienters in their number of premature nosepokes during the ready period ($p > 0.2$) nor in the percent of trials with correct responses ($p > 0.4$).

An orienting classification (Orienters vs. Nonorienters) x attentional challenge (baseline, 100ms, variable ready period, blinking light) repeated measures ANOVA was performed to assess whether animals showed behavioral changes during the three different attentional challenges compared to their own baseline training and whether the pattern was different between Orienters and Nonorienters. In terms of premature

responses during the ready period (Figure 4.4A), there was only a significant main effect of attentional challenge, $F(3, 42)=19.74$, $p<0.0001$. A post-hoc Bonferroni test revealed that both Orienters ($p<0.001$) and Nonorienters ($p<0.05$) displayed significantly fewer premature responses when the house light blinked ("distractor"). Orienters and Nonorienters displayed comparable premature responses in all of the attentional challenges as evidenced by no main effect of orienting classification and no interaction effect. In terms of the percent trials with correct nosepoke responses (Figure 4.4B), there was a significant main effect of attentional challenge, $F(3, 42)=10.63$, $p<0.001$, but no main effect of orienting classification ($p>0.5$). However, there was a marginally significant interaction effect of orienting classification and attentional challenge, $F(3,42)=2.53$, $p=0.07$. A post-hoc Bonferroni test revealed that Orienters were significantly less accurate when the ready period varied to 1, 5, 9 s from constant 5 s ($p<0.001$) and when the house light signaling ready period blinked instead of being steady ($p<0.05$). For Nonorienters, however, only the variable ready period resulted in significantly less accurate nosepoke responses ($p<0.05$).

A regression analysis showed that normalized orienting score predicted greater accuracy of responding in the baseline test, $R^2 = 0.32$, $F(1, 14) = 6.56$, $p < 0.05$ (Figure 3.4C left panel) even though the group ANOVA analysis revealed no significance. Furthermore, rats with higher orienting scores tended to be more distracted by a blinking house light, although this relationship was only marginally significant, $R^2 = 0.23$, $F(1, 14) = 4.26$, $p= 0.058$ (Figure 4.4C right panel). Further correlation analyses using the AUCs of delay discounting and risky decision-making showed no relationship between these two earlier tasks and the performance during any of the 5-choice baseline or attentional challenges.

4.4.2. Experiment 2

4.4.2.1. Light-food pairings

In terms of OR scoring, instead of using the timed sampling described in Experiment 1, we used the probability of OR occurrence in a given trial. As shown in Experiment 1, both measures produce comparable results and are highly correlated (R^2 = 0.88). As in Experiment 1 there was a general acquisition of conditioned OR and conditioned food cup approach. However, as in Experiment 1, only a subset showed persistent OR and rats were divided into Orienters (n=7) and Nonorienters (n=7) based upon their median number of OR within the CS1 during the last 8 trials of training. An orienting classification x trial block repeated ANOVA of OR revealed a significant interaction effect, $F(6, 72)= 5.88$, $p <0.0001$, indicating that Orienters acquired a more robust OR. By the end of the training (last 8 trials), there was a significant difference in OR between Orienters and Nonorienters, $t(12)=4.33$, $p=0.001$. An orienting classification x trial block repeated ANOVA of food cup approach behavior during CS2 indicated that both Orienters and Nonorienters acquired food cup approach similarly. There was only a significant main effect of trial block, $F(6, 72) = 7.30$, $p < 0.001$ with neither an interaction effect, $F(6, 72) = 0.75$, $p > 0.6$, nor a main effect of orienting classification, $F(1,12)=4.08$, $p>0.05$.

4.4.2.2. Open field and light-dark emergence tasks

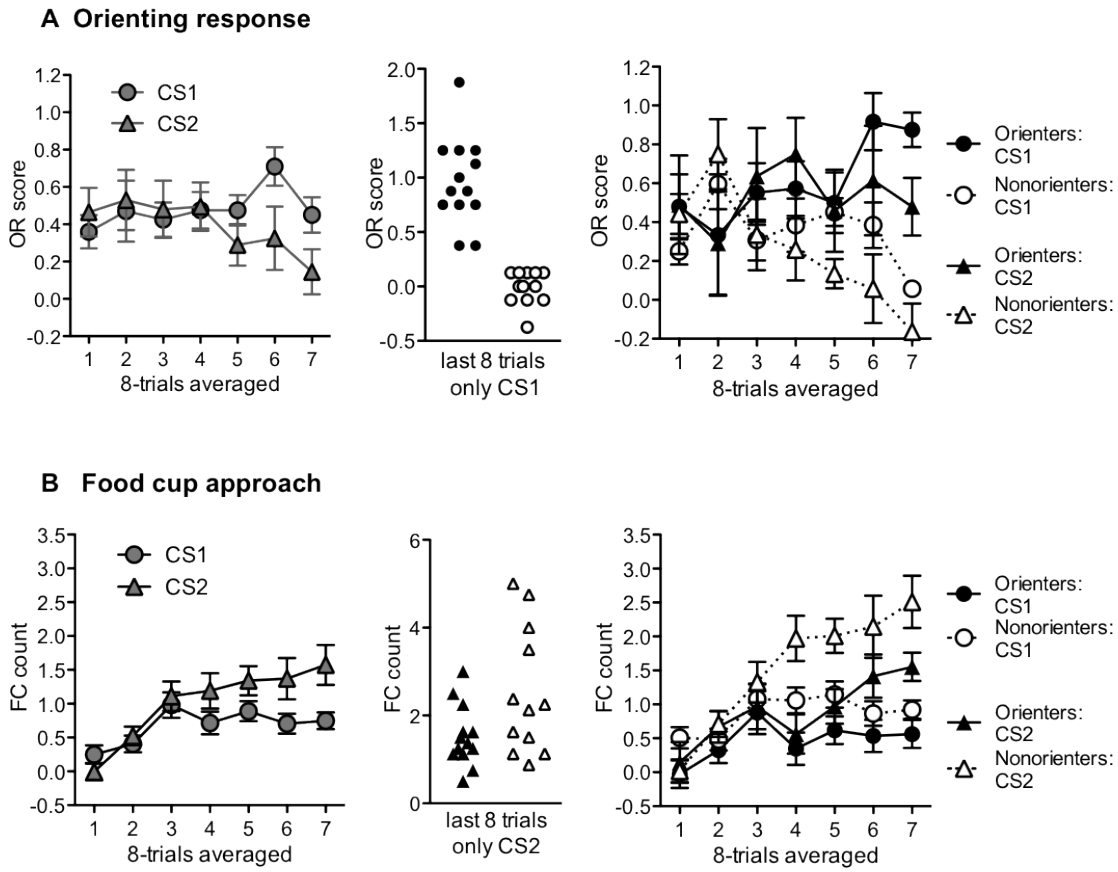
During the general open field activity measures on the first day, Orienters and Nonorienters did not differ in their activity levels, 3600 (± 567) and 3800 (± 663) cm in distance (± SEM) traveled, respectively. By the second day, the overall activity level was reduced, $F(1,12)=18.48$, $p<0.001$, but there was no difference between the two groups

(Orienters 2400 ± 227 cm and Nonorienters 2600 ± 543 cm). After being confined to the dark compartment for 10 mins on the third day, the animals were returned to the dark compartment on the fourth day but were free to enter the light field. As seen in Figure 4.5A, Orienters did not differ from Nonorienters in their activity level, in either the light or dark compartments, as seen by t-tests comparing mean ambulatory distance of Orienters and Nonorienters in dark compartment, $t(12) = 0.09$, $p = 0.923$, and light compartment, $t(12) = 0.74$, $p = 0.47$. Orienters also did not show a greater preference than Nonorienters for exploring the novel, bright compartment, as measured by a comparison of the mean amount of time they spent there, $t(12) = 0.84$, $p = 0.42$ (Figure 4.5B). However, Orienters entered the novel open field significantly sooner than Nonorienters (log-rank test $p < 0.05$). All Orienters exited the dark compartment within the first 45 s while most Nonorienters took much longer, including one that never left the dark compartment during the 10-min test session (Figure 4.5C).

4.4.3. Experiment 3

As in experiment 1, based on their average OR scores within CS1 during the last eight training trials, rats were divided into two groups. Rats scoring above the median OR score were classified as Orienters (n= 8), while those rats that scored below the median score were classified as Nonorienters (n=8). An orienting classification x two-way repeated ANOVA (last 5 training sessions x 5 shock sessions) revealed no significant effect of orienting, no effect of shock risk, and no interactions (p's >0.1). Rats chose the lever associated with shock on only 14% of trials, indicating that when the two levers produced rewards of equal size, both Orienters and Nonorienters preferred the lever that was not associated with risk of shock.

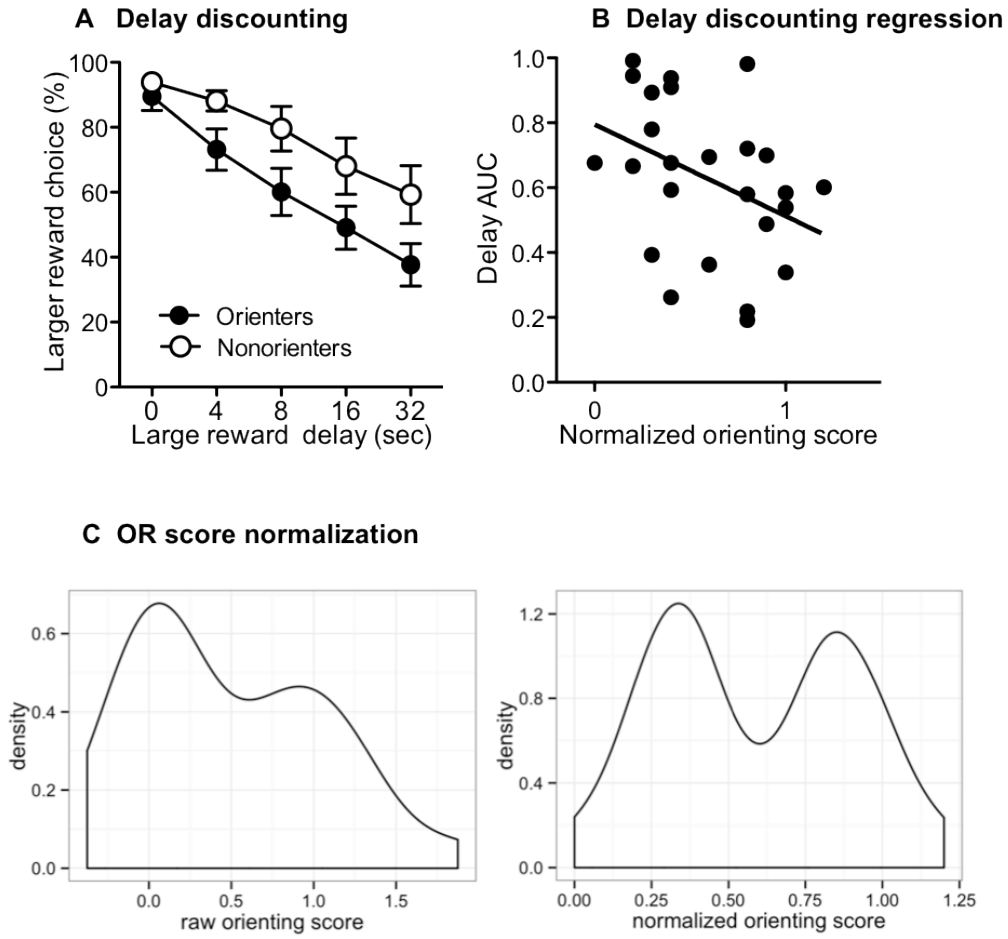
Figure 4.1. Acquisition of orienting and food cup approach



(A, left panel) Mean (±SEM) conditioned OR scores of all the rats from experiment 1 is shown as during the 1st half of light CS presentation (CS1) and the 2nd half of light CS presentation (CS2). Overall, there is more orienting during CS1. (A, middle panel) Individual orienting scores during CS1 from the last 8 trials. Filled circles represent the rats classified as Orienters ($\geq$ median) and the open circles represent the rats classified as Nonorienters. (A, right panel) Mean (±SEM) conditioned OR scores oranized based on the orienting classification (B, left panel) Mean (±SEM) conditioned food cup approach counts of all the rats from experiment 1 is shown as during the 1st half of light CS presentation (CS1) and the 2nd half of light CS presentation (CS2). Overall, there is

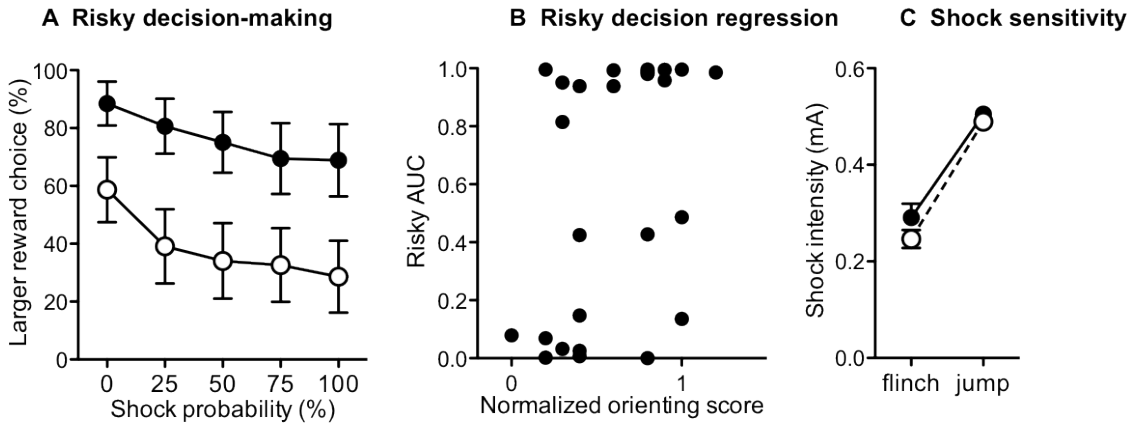
more food cup approach during CS2. (B, middle panel) Individual food cup approach counts during CS2 from the last 8 trials. Filled triangles represent the rats classified as Orienters and the open triangles represent the rats classified as Nonorienters. (A, right panel) Mean (±SEM) conditioned food cup approach oranized based on the orienting classification.

Figure 4.2. Delay discounting



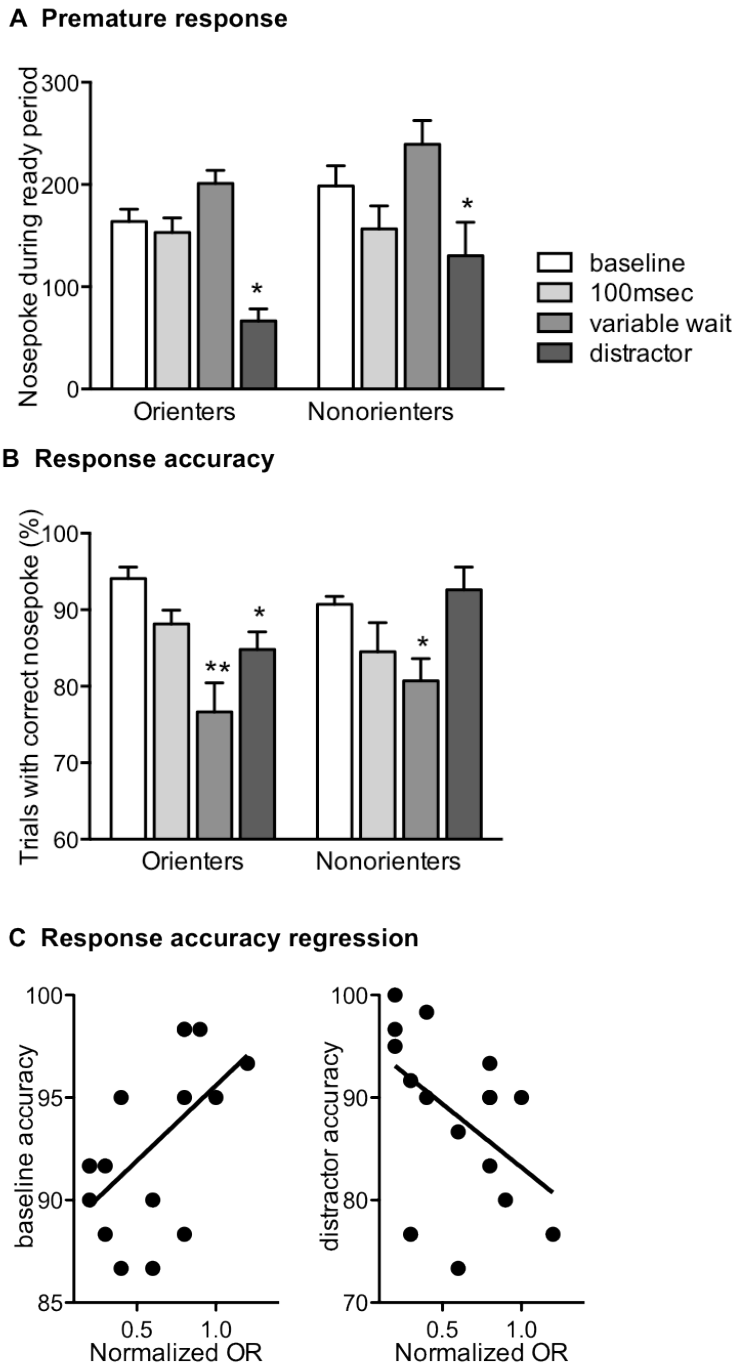
(A) Mean percent choices (±SEM) of Orienters and Nonorienters for the larger reward associated with increased delay over the last 5 days of training. Orienters chose larger reward significantly less compared to Nonorienters as the delay increased. (B) A plot of the regression comparing normalized orienting scores to delay discounting AUC. There was a tendency for orienting score to predict performance in delay discounting (p=0.06). (C) The probability density plots of raw OR scores from the last 8 trials (left panel) and the log-normalized scores (right panel) which were used for regression analyses. The normalized plot demonstrates bimodal distribution of the OR scores.

Figure 4.3. Risky decision-making



(A) Mean percent choices (±SEM) of Orienters and Nonorienters for the larger reward associated with a shock probability over the last 5 days of training. Orienters chose larger reward associated with shock significantly more compared to Nonorienters. (B) A plot of the regression comparing normalized orienting scores to risky decision-making AUC. The ranked orienting score predicted performance in the risky decision-making ($p<0.05$). (C) Mean intensity of footshock (±SEM) that evoked flinch and jump responses from the Orienters and Nonorienters.

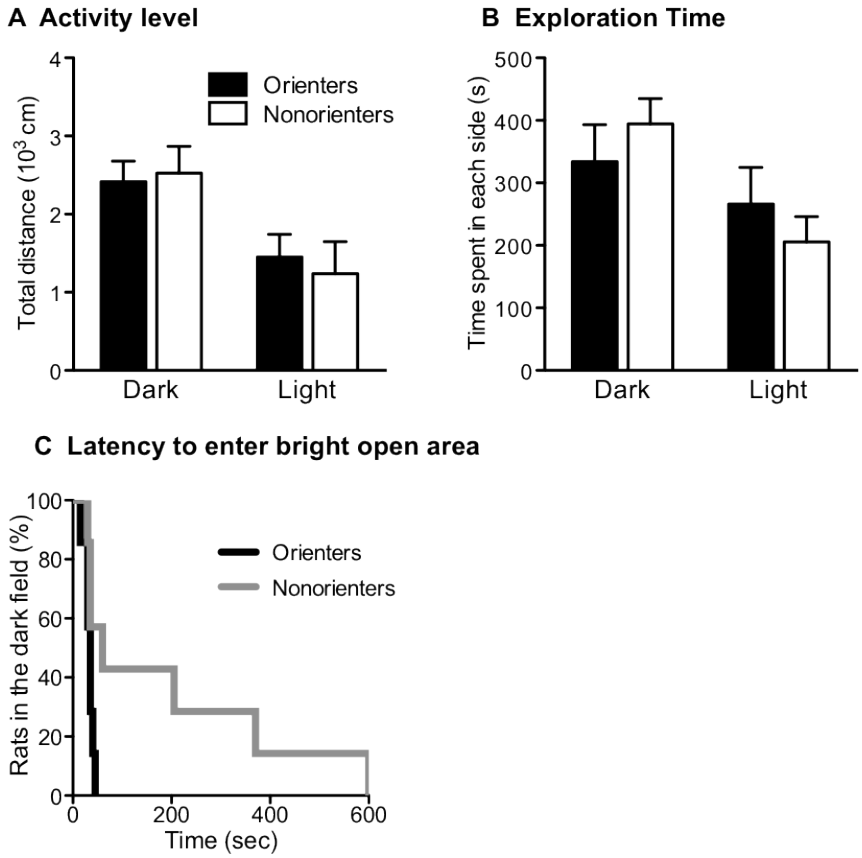
Figure 4.4. 5-choice serial reaction time task



(A) Mean (±SEM) number of nosepokes made during the ready period and (B) mean (±SEM) percent trials with correct nosepoke response in the 5CSRTT sessions. Data

from the last baseline (500 ms port light) and three attentional challenges are presented. 100 msec, port light was reduced from 500 msec to 100 msec; variable wait, the typical 5 sec ready period was varied among 1, 5, 9 sec; distractor, the typical steady house light signaling wait period blinked for the 5 sec ready period. **$p<0.001$ & *$p<0.05$ compared to their own baseline level. (C) Regression analyses of normalized orienting score and baseline accuracy (left panel) and accuracy during the house light blink (distractor) test (right panel).

Figure 4.5. Open field



(A) Mean (±SEM) distance traveled by Orienters and Nonorienters within the black insert (dark) and the open field (light). There was no difference in the activity level between Orienters and Nonorienters. (B) Mean (±SEM) time spent by Orienters and Nonorienters within the black insert (dark) and the open field (light). There was no difference in preference between Orienters and Nonorienters. (C) Kaplan-Meier plots of the percentage of Orienters vs. Nonorienters that have not yet exited the familiar dark compartment (and entered the novel light compartment), as a function of time. Graph represents latency to enter the open area. Orienters entered the bright open field much faster than Nonorienters, $p<0.05$.

4.5. DISCUSSION

The current studies sought to characterize impulsive behaviors and related traits as well as the attentional function of rats that show robust conditioned orienting (Orienters) in an appetitive Pavlovian conditioning paradigm. Our results suggest that Orienters, as compared to Nonorienters, make more impulsive and risky choices, are more distractible when faced with an attentional challenge, and are more willing to approach a novel open environment. However, Orienters did not differ from Nonorienters in their general activity level, their overall preference for exploring a novel illuminated space over a familiar dark one, or in their impulsive premature responding.

Impulsivity can be described as a cluster of related but independent phenomena including impulsive action, impulsive choice, and riskiness in decision-making (Evenden, 1999). Our data show that Orienters are more apt to make impulsive choices during the delay discounting task and to risk foot shock in order to receive a larger reward. However, the results from the 5CSRTT provide little evidence that Orienters and Nonorienters differ in their premature responses, a measure of impulsive action. Earlier studies that examined the relationship between sign-tracking (i.e., lever approach behavior) and impulsivity (Flagel et al., 2010; Lovic et al., 2011) reported that sign-tracking animals showed reduced impulsive choice in a delay discounting task and increased impulsive action, as measured by premature responses. Their premature responses were measured using a 2-choice serial reaction time task or a DRL task (differential reinforcement of low rates of responding) rather than the 5CSRTT task used here. The key difference, however, is the resulting consequence of premature responses. In the aforementioned studies, premature or incorrect responses resulted in timeout or reset of the trial. In the current study, premature responses were not punished and rats were allowed to make a correct response following a premature or incorrect response.

Holland and colleagues (Holland et al., 2000; Holland, 2007; Maddux et al., 2007; Maddux and Holland, 2011) developed this protocol in order to equate the total number of trials experienced by each animal, as the typical protocol of punishing premature responses can result in vast differences in the numbers of completed trials between animals. Thus, even though the current protocol provides an unbiased and uninterruped measure of premature responses, it may not be ideal for measuring inhibitory control because animals are not encouraged to inhibit premature responses. It is possible that differences in impulsive action between Orienters and Nonorienters might have been observed under circumstances in which they were encouraged to inhibit premature responses.

A discrepancy was also observed in the relationship between impulsive decision making and sign-tracking. A delay discounting task was used in all studies, but reports from both Flagel et al. (2010) and Lovic et al. (2011) indicate that sign-trackers make fewer impulsive choices. Our current study shows more impulsive choices among Orienters. Tomie et al. (1998) also reported sign-trackers made more impulsive choices, but their results are mainly driven by the potentially learned lever bias regardless of reward magnitude and do not necessarily address the relationship between sign-tracking behavior and impulsivity. However, lever bias doesn't seem to play any role in our reported results. The delay discounting and the risky decision-making tasks were conducted in the same conditioning chambers and for each subject the lever that led to the large reward either with delay (in the delay discounting task) or with the risk of footshock (in the risky decision-making task) was in the same location. It seems possible that this protocol might predispose rats to consistently choose one lever over the other in both tasks. However, our results indicate the opposite – Orienters preferred the lever

yielding the small reward in the delay discounting task while they preferred the lever yielding the large reward (i.e., opposite lever) in the risky decision-making task.

Conditioned orienting and lever-approach behavior, two forms of cue-elicited behavior, may reflect different behavioral phenotypes with distinct neural mechanisms. The central nucleus of the amygdala (CeA) is known to play a crucial role in mediating conditioned orienting (Gallagher et al., 1990). Our recent study also highlights the importance of OR behavior and CeA function in mediating CS-associated memory (Olshavsky et al., 2013). However, several studies suggest that the CeA may be minimally involved in sign-tracking to a lever. In a study by Flagel and colleagues (2011), no differences in *c-fos* mRNA expression were observed in the CeA of sign-trackers (rats that approached a lever CS) and goal-trackers (rats that approached the food cup). Furthermore, CeA opioid stimulation enhanced both lever-approach and food cup approach behavior in sign- and goal-trackers, respectively (DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2009). Finally, Chang et al. (2012) reported that lesions to the CeA did not interfere with autoshaped lever-pressing behavior. While some specific circuitries may not be shared by these two types of cue-elicited behaviors, dopamine neurotransmission appears to be involved in both forms of cue-elicited behaviors (El-Amamy and Holland, 2007; Han et al., 1997; Lee et al., 2005, 2010, 2011; DiFeliceantonio and Berridge, 2012; Flagel et al., 2011b). Dopamine in cortico-striatal circuitry has also been implicated in impulsive and risky decision-making processes, as well as the individual differences in these behaviors (Cardinal, 2006; Dalley et al., 2008; Floresco et al., 2008; Jentsch and Taylor, 1999; Simon et al., 2011). More work is needed to understand individual differences in sign-tracking behavior and its relationship with impulsivity. Our current work suggests that conditioned orienting and lever-approach behavior might not share similar mechanisms for predicting impulsive behaviors.

The 5CSRTT task provides the opportunity to examine potential differences in attentional function between Orienters and Nonorienters. Previously, Robinson et al. (2009a) reported that rats displaying both impulsive action (premature responding) and choice (in delay discounting) were less accurate during the 5-choice task when compared to less impulsive rats. A recent study reported that rats displaying prepotent lever-approach behavior performed worse during a 2-choice task (Paolone et al., 2013). Here we report that even though both Orienters and Nonorienters performed similarly during the baseline test, Orienters were generally more impaired when faced with attentional challenges. The relationship between impulsivity and attention is particularly important for its relevance in understanding Attention Deficit Hyperactivity Disorder, ADHD (Russell, 2007). In another study investigating the role of orienting behavior in understanding impulsivity and attentional function, spontaneously hypertensive rats (a commonly used animal model for ADHD with a hyperactive and impulsive phenotype) showed enhanced orienting to a novel light stimulus and subsequent failure to habituate orienting to the light after repeated presentations (Hopkins et al., 2009; Robinson et al., 2012). In the present study, Orienters (which made more impulsive and risky choices) were also more likely to be distracted during an attentional task. However, we also showed that impulsive action as measured by premature responding was not necessarily related to attentional distractibility. It is well known that neural and pharmacological manipulations can influence impulsivity, particularly premature responding, and different aspects of attention function independently (Robbins, 2002). It is therefore likely that the relationship between impulsivity and attention is specific to the subtypes of these cognitive domains, a hypothesis that deserves further investigation.

Finally, we reported that Orienters took less time to leave a familiar dark environment and venture into a novel illuminated field, despite the fact that Orienters and

Nonorienters ultimately spent comparable time and had similar activity levels in the illuminated field. Overall, both groups spent more time in the familiar dark compartment. Molander et al. (2011) reported that high-impulsive rats (showing more premature responses) spent more time exploring a novel environment than a familiar one and that a trend existed for low-impulsive rats to spend more time in the familiar environment. Our results show little evidence that Orienters (with high preference for impulsive and risky choices) prefer a novel environment as measured by dwell time and activity levels. However, whereas Orienters did not necessarily display a preference for the novel environment, they had shorter latencies to enter it initially. This difference might reflect the greater impulsive and risky decision-making shown by Orienters, i.e. an impulsive entry into the new "risky" place (followed by a hasty retreat back into the familiar "safe" compartment) vs. a considered entry after an extended evaluation of the new environment (e.g., by peeping out of the opening from the safety of the familiar environment). Previous work has demonstrated a rat behavioral phenotype characterized by hyperactivity in novel environments with increased exploration of *and* impulsive entries into the light compartment in the light-dark test (Kabbaj, 2004; Shumake et al., 2005). Thus, the traits of novelty-evoked hyperactivity, elevated risk taking, and impulsivity may co-occur, but the current results show impulsive-like exit latencies for Orienters without accompanying hyperactivity or prolonged risk taking. This suggests that these traits are in fact dissociable and that Orienters may offer a model of impulsive behavior isolated from other associated traits.

It should be noted that the current study did not counterbalance the order of the tasks administered. The rats were first categorized as Orienters and Nonorienters based on their performance during classical appetitive conditioning. Then, they were tested in the order of the delay discounting task, the risky decision making, and the five-choice

task. In terms of the dark-light emergence task, the rats also received the appetitive conditioning first. Thus, the current study is limited in addressing whether the earlier tasks could have potentially influenced the subsequent tasks. However, in conjunction with the findings from our earlier studies, we believe it is unlikely that the individual differences seen in the risk decision-making and five-choice tasks are entirely due to the different patterns of reinforcement obtained during the delay discounting task. In our current study, we did not see any significant correlation between delay discounting behavior and the other two tasks (i.e., risky decision making and five-choice task). In our earlier study (Simon et al., 2009), there was also no correlation between delay discounting and risky decision-making. It should be noted that the risky decision making task was administered prior to the delay discounting task in the Simon et al., 2009 study. Furthermore, in the same study, the rats were tested again for risky decision-making after the delay discounting task, and showed no changes in risky decision making pre- and post-delay discounting task. In terms of the dark-light open field test, it is unlikely that the experience of appetitive conditioning (and the additional extinction and spontaneous recovery tests) resulted in individual differences. In our recent study with a much larger sample of 66 rats (Olshavsky et al., 2013a), the same pattern emerged even when the rats were tested in the dark-open field first prior to the classical appetitive conditioning that was used to classify the rats Orienters and Nonorienters retrospectively. It should also be noted that the rats in Olshavsky's study were in regular light cycle and on restricted feeding, unlike the rats in the current study.

Cue-elicited behavior, whether it entails approaching a lever or orienting to a light, can be considered a maladaptive behavior in that animals are performing a response which may delay or even preclude delivery of reward. In their work *The Misbehavior of Organisms*, Breland and Breland (1961) described raccoons that lost interest in their

reward after becoming preoccupied with the objects with which it was paired. In addition, our data show an Orienter phenotype more apt to make impulsive and risky decisions and more likely to be distracted. While our study has shown orienting to be related to impulsivity, risk-taking, and to some extent attention, the exact nature of how these traits parallel or diverge within individuals remains to be examined.

Chapter 5: Orienters' and Nonorienters' responses to amphetamine

5.1. ABSTRACT

There is ample evidence to suggest that individuals differ in their subjective response to dopamine agonist exposure and it has been suggested that an enhanced subjective response indicates a sensitized mesolimbic dopamine system (Everitt and Wolf, 2002; Kalivas, 2004; Robinson and Berridge, 2001; Wise, 2004). In our lab, we have identified behavioral phenotypes that show robust cue-directed conditioned responses during light-food pairings (Orienters) and those that do not (Nonorienters). As these cue-directed responses are known to be dependent upon dopamine function, the current study aimed to investigate how these behavioral phenotypes might differ in their subjective response to d-amphetamine, a dopamine agonist. First, we showed that when administered amphetamine within the home cage, Orienters vocalize more than Nonorienters. Also, while both Orienters and Nonorienters prefer an amphetamine-paired context to one associated with saline administration, Orienters vocalized more while in that context. We suggest that these differences in subjective response to amphetamine indicate differences in the mesolimbic dopamine function of Orienters and Nonorienters.

5.2. INTRODUCTION

Individuals vary in their subjective response to amphetamine exposure and drug users who experience an enhanced subjective response may be more susceptible to drug addiction and failed attempts at abstinence (De Wit and Phillips; 2012). Traditionally subjective response in humans has been quantified by subjects' own vocal reports of how

enjoyable they find the drug experience, whether they would take it again, and whether they would pay for it. However, study of this measure in non-human animals has been difficult. Recently, ultrasonic vocalizations have garnered interest as a potential indicator of the emotional state created by drugs of abuse (Ahrens et al., 2009). It has been widely suggested that in rats, vocalizations within the 30-90 kHz range are indicators of positive affect. These calls, with a typical average frequency of 50 kHz, are emitted when juvenile rats anticipate the opportunity to play, when rats are tickled, mating, consuming food, and self-stimulating with electrical pulses or addictive drugs, such as amphetamine and cocaine (Knutson et al., 1998; Burgdorf and Panksepp, 2001; Panksepp and Burgdorf, 2000; Schwarting et al., 2007; Bialy et al., 2000; Burgdorf et al., 2000; Wintink and Brudzynski, 2001; Maier et al. 2012). In contrast, vocalizations in the 20-30 kHz range ("22-kHz calls") are believed to indicate negative affect and are emitted after exposure to predatory odors, social defeat, or cues associated with foot shock (Borta et al., 2006; Brudzynski, 2001; Knutson et al., 2002).

This lab has previously described behavioral phenotypes that show robust cue-directed responding during Pavlovian conditioning (Orienters) and those that do not (Nonorienters) (Olshavsky et al., 2013a,b). We have also shown that Orienters and Nonorienters differ in their delay discounting, risky decision-making, and attentional processing, all behaviors known to be dependent upon amygdalo-nigrostriatal dopamine function (Chapter 4 of this dissertation). The goals of the current experiments were three-fold. First, as the conditioned orienting response is dependent upon amygdalo-nigrostriatal dopamine circuitry, we hypothesized that Orienters and Nonorienters might have fundamental differences in the function or sensitivity of this circuitry which could be reflected under dopamine agonist challenge. Secondly, while Orienters and Nonorienters differ in their conditioned responses to discrete cues, it is unknown how

they might differ in their responsiveness to context conditioning. Finally, the previous work reported in this dissertation has shown that Orienters are more apt to make risky and impulsive choices and are more susceptible to distraction, both characteristics used to describe humans vulnerable to drug use and abuse disorders. For these reasons, the current experiments were designed to investigate how Orienters and Nonorienters might differ in their response to dopamine agonism, as measured by both ultrasonic vocalizations and preference for an amphetamine-paired context.

5.3. EXPERIMENT 1: ULTRASONIC VOCALIZATION IN THE HOMECAGE

5.3.1. Subjects

Fourteen male Long-Evans rats (Charles River) weighing 250-275 g upon arrival were singly housed in a 10-hour light/14-hour reverse cycle with the lights off at 10 am. Food and water were available ad libitum during acclimation to the colony. Prior to beginning training, rats were reduced to 90% their free-feeding weight, and this weight was maintained throughout Pavlovian conditioning. Rats were fed ad libitum diet during amphetamine administration and USV recording. All experiments were conducted according to the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

5.3.2. Methods

5.3.2.1. Pavlovian Conditioning

Pavlovian conditioning took place within eight individual conditioning chambers (30.5 cm W x 25.4 cm D x 30.5 cm H, Coulbourn Instruments). Each chamber had

aluminum sidewalls and ceiling, with clear acrylic front and back walls. The floor was made of stainless steel rods (0.5 cm in diameter, spaced 1.0 cm apart). The food cup was located on the right-hand wall of the chamber, 2.5 cm above the floor. Nose-poke entry into the food cup was detected by an infrared beam at the opening. A 2-w white light was mounted 20 cm above the food-magazine and its illumination served as a CS signaling grain pellet delivery (Test Diet, 45 mg). On the wall opposite the magazine were five ports. The ports remained inactive during the conditioning procedures. Each chamber was enclosed in a light- and sound-attenuated box (58.4 cm x 61 cm x 45.7 cm) where a ventilation fan provided masking noise. A video camera was mounted within each box and images were recorded during behavioral training and testing. Training began with a session in which rats learned to retrieve grain pellets from the food cup. A total of 30 pellets were delivered at a variable interval (averaging 60 s) over the 30-min session. The following day, rats began conditioning. The first conditioning session consisted of two parts: habituation of the unconditioned response to light and then CS-US pairings. The first half of the session consisted of 8 illuminations of the light, and served to habituate any unconditioned orienting to this novel stimulus. During the second half of the session, 8 light presentations co-terminated with the delivery of a grain pellet to the food cup. For the next three days of conditioning, sessions consisted of 16 light – food pairings with a variable intertrial interval (ITI) averaging 120 seconds.

Previous work has shown that when presented with a 10 s light CS that predicts pellet delivery into a food cup, rats typically display two readily observable conditioned responses: conditioned rearing and conditioned food cup approach. Conditioned rearing typically occurs during the first five seconds of the CS (CS_1) and food cup approach response occurs during the last five seconds (CS_2) (Holland, 1977). It is important to differentiate conditioned rearing to a light predictive of food from the unconditioned

orienting response that occurs whenever a rat is presented a novel light stimulus. In order to habituate unconditioned orienting, all rats were exposed to the light CS prior to the start of training. This pre-exposure resulted in habituation of the unconditioned rearing response so that any rearing observed during CS illumination could be interpreted as the result of the CSs association with the US.

For both experiments, rearing was scored by a blind observer from DVD recordings of the training sessions. A rearing response was defined as a lifting of both forelimbs from the floor of the conditioning box, and did not include grooming behavior. Because during the ITI the conditioning chamber was completely dark and the illumination of the CS provided diffuse illumination of the entire chamber, rearing behavior in any direction was included in the reporting of conditioned responses. Rearing behavior was sampled every 1.25 seconds during the 5 seconds prior to CS onset (pre-CS) and during the 10 s CS. This provided 12 observation points. To account for within-groups variation in baseline rearing, we report the difference in CS_1 and pre-CS responding (responding during the 5 seconds prior to the CS). Rearing results are reported as counts, subtracting the number of observation points a rear was recorded during pre-CS from the number recorded during CS_1. Food cup approach is reported as bouts of nosepokes into the magazine as measured by the infrared beam. We report the difference in CS_2 and pre-CS food cup responding.

Twenty-four hours after Pavlovian training, all rats underwent an extinction session, 24 hours after extinction they were tested for long-term memory, and 21 days following the long-term memory test they were tested for spontaneous recovery. The data from these tests are reported in Chapter 2. After the test for spontaneous recovery, rats' food and water were both available ad libitum.

5.3.2.2. Amphetamine administration and USV recording

One month after the test for spontaneous recovery, rats were given an intraperitoneal (IP) injection of saline and returned to the homecage. The home cage was immediately placed within a sound-attenuating chamber and ultrasonic vocalizations were recorded with a microphone (Avisoft, Germany) mounted to the lid of the cage. Recordings lasted 30 minutes. Twenty-four hours after the saline injection, all rats were given an (IP) injection of 2 mg/kg d-amphetamine (dextroamphetamine, Sigma-Aldrich) dissolved in saline and again recorded for 30 minutes within the same apparatus. Recordings were analyzed using Avisoft-SASlab Pro software (Avisoft Bioacoustics; Berlin, Germany). Spectrograms were generated and vocalizations were counted by visual analysis of spectrogram images. Vocalizations falling between 30-95 kHz were termed 50-kHz calls and were categorized as either frequency modulated (FM) or flat based upon the presence or absence of sinusoidal fluctuations in frequency (Ahrens et al., 2009; Simola et al., 2009). Vocalizations in the 20-30 kHz range were termed 22 kHz calls (Wright et al. 2010; Brudzynski, 2001).

5.3.3. Results

5.3.3.1. Pavlovian conditioning

Rats were classified as Orienters and Nonorienters based upon the number of OR counted during individuals' last 8 trials of training. Rats scoring above the median number of OR counts (median = 0.5) were classified as Orienters (n = 7), while those scoring below were classified as Nonorienters (n = 7). Orienters acquired robust conditioned orienting behaviors as training progressed, as evidenced by an orienting classification x trial repeated measure ANOVA revealing an interaction between the two

factors, $F(6, 72) = 5.88$, $p < 0.001$. However, the groups acquired comparable food cup approach behaviors. An orienting classification x trial repeated measure ANOVA of food cup approach behavior revealed only a main effect of trial, $F(6, 72) = 7.30$, $p < 0.001$.

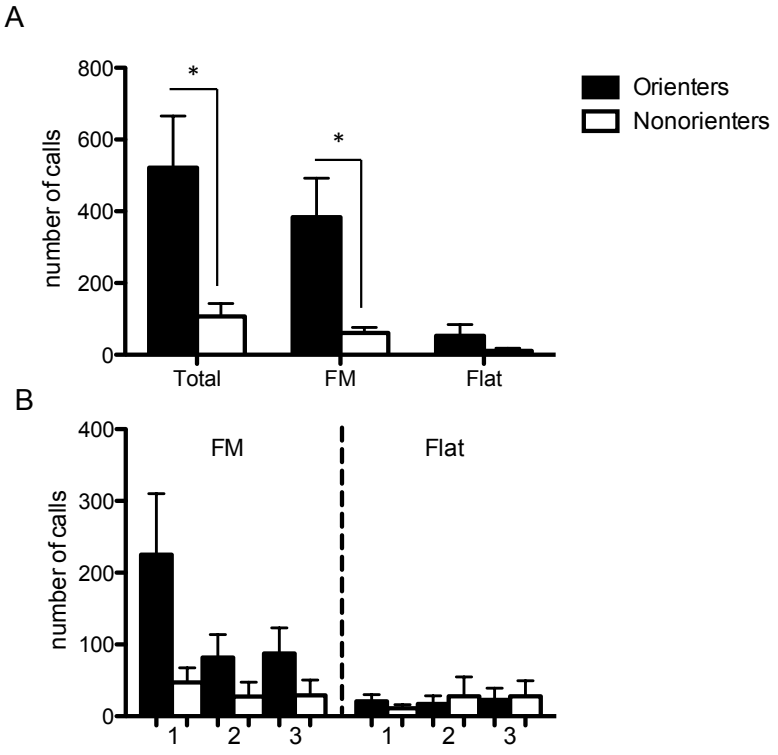
5.3.3.2. Amphetamine administration and USV recording

Orienters and Nonorienters did not differ significantly in the number of vocalizations after saline injection (Orienters mean = 36, Nonorienters mean = 109, $p = 0.46$). Further analysis of 50-kHz vocalization type (flat vs. FM) and timing of calls (1^{st}, 2^{nd}, or 3^{rd} 10-minute block) showed that both call types were emitted equally throughout the baseline session. These findings were supported by an orienting classification x call type x session block repeated measures ANOVA showing no significant effects (all p's > 0.2). No 22-kHz vocalizations were observed during this baseline session.

In order to account for individuals' baseline differences in vocalization, the number of flat and FM calls made during the baseline session was subtracted from the number of calls made during the amphetamine test session. During this session, Orienters called more than Nonorienters (Figure 5.1A) and this effect was driven by the Orienters' increase in FM vocalizations, with the largest number of FM vocalizations occurring within the first 10 minutes of the session (Figure 5.1B). These results are supported by an orienting classification x call type x session block repeated measures ANOVA (main effect of orienting: $F(1, 12) = 7.75$, $p = 0.015$; main effect of call type: $F(1, 12) = 15.21$, $p = 0.002$; orienting x call type interaction: $F(1, 12) = 8.27$, $p = 0.014$). Additionally, while flat calls were remained infrequent throughout the session, FM calls were most frequent during the first 10-minute block and decreased over time (call type x

session block interaction, $F(2, 24) = 5.13$, $p = 0.014$). No 22-kHz calls were observed during the amphetamine session.

Figure 4.1. Vocalizations after amphetamine in the home cage



(A) Mean (±SEM) number of total calls, frequency modulated (FM) calls, and flat calls of Orienters (black bars) and Nonorienters (white bars) during the 30 minutes following amphetamine injection. (B) Mean (±SEM) FM and flat calls during 1st, 2nd, and 3rd 10-minute blocks of the 30-minute session.

5.4. EXPERIMENT 2: CONDITIONED PLACE PREFERENCE

5.4.1. Subjects

Thirty-four male Long-Evans rats (Charles River Laboratories) weighing 250-275 g upon arrival were singly housed in a 12-hour light/dark cycle with the lights on at 7 am. Food and water were available ad libitum during acclimation to the colony. Prior to inclusion in this experiment, all rats were fear conditioned using tone and foot shock pairings and all rats' conditioned fear was extinguished (results reported in Olshavsky et al., 2013). Prior to beginning appetitive conditioning procedures, rats' weights were reduced to 90% their free-feeding weight. This weight was maintained only during the Pavlovian conditioning portion of the experiment; during the conditioned place preference portion food and water were available ad libitum. All experiments were conducted according to the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

5.4.2. Methods

5.4.2.1. Pavlovian Conditioning

Pavlovian conditioning took place within the same eight conditioning chambers described in Experiment 1 and procedures were identical to those described above. As in Experiment 1 rats were classified as Orienters and Nonorienters based upon their median OR score during the final 8 trials of training.

5.4.2.2. Conditioned Place Preference (CPP)

CPP training and testing took place within 4 shuttle boxes, each contained within a sound-attenuating chamber. The shuttle boxes consisted of clear acrylic front and back

walls and aluminum side walls. An aluminum wall with retractable door divided the shuttle box into two halves. In one half (black side) black paper was hung outside the acrylic walls. In the other half (white side) white paper was hung outside the acrylic walls. Both sides of the chamber were illuminated with a 2-W light; a red bulb was mounted 20 cm above the floor in the white side and a white bulb was mounted in the black side. A video camera mounted above the black side recorded time spent there. Time spent within each side was scored by a blind observer from recordings of the test sessions.

CPP procedures consisted of 3 phases: baseline test, conditioning, and post-tests. For the baseline test, the retractable door was open. All rats were initially placed into the black side of the box and were allowed 15 minutes of free access to both sides in order to assess individuals' baseline preference for either the black or white side. While individuals showed preferences for a particular side, there was no apparatus bias; the average time spent in the black and white sides during this baseline test was not significantly different. There were also no group differences; Orienters and Nonorienters showed no difference in the time spent in the black side during baseline test (Orienters' mean = 486 s, Nonorienters' mean = 442 s). Based on how much time each subject spent in the black or white side, a preference was determined for each rat. For example, if during baseline testing a rat spent 490 s in the black side and 410 s in the white side, the rat's preference was for the black side. Twenty-four hours after baseline testing and preference determination, all rats received an IP injection of saline and were restricted to their preferred side of the shuttle box for 30 minutes (Conditioning day 1). The following day rats received either a 1 mg/kg or 2 mg/kg dose of d-amphetamine (dextroamphetamine, Sigma-Aldrich) via IP injection and were placed in their non-preferred side for 30 minutes. Conditioning days 3 and 4 were identical to days 1 and 2.

For example, if an individual preferred the black side during baseline test, saline was paired with the black side on days 1 and 3 and amphetamine administration was paired with the white side on days 2 and 4. Twenty-four hours after conditioning day 4, rats were tested for preference. This test (Test 1) was identical to the baseline test; rats were initially placed within in the black side and were allowed 15 minutes free access to both sides of the shuttle box. Eleven days after, rats were again tested for preference using identical procedures (Test 2).

5.4.3. Results

5.4.3.1. Pavlovian conditioning

During the appetitive training sessions, there was an overall acquisition of conditioned OR. However, a subset of rats did not display persistent conditioned OR. Thus, based on their average number of OR bouts during the last eight trials of training, rats were divided into two groups. Rats scoring at or above the median number of OR bouts (0.44 bouts/trial) were classified as Orienters (n= 19), while those rats that scored below the median score were classified as Nonorienters (n=15). In support of these findings, an orienting classification x trial repeated ANOVA of OR revealed a significant effect of trial, $F(13, 416) = 5.251$, $p < 0.001$, and an orienting classification x trial interaction, $F(13, 416) = 1.806$, $p < 0.05$.

Both Orienters and Nonorienters increased conditioned food cup responses as training progressed, as evidenced by a main effect of trial in an orienting classification x trial ANOVA of food cup responding, $F(13, 416) = 5.251$; $p < 0.001$. Additionally, there was a trend toward the end of training for Orienters to show a slight reduction in food cup approach, as compared to Nonorienters; however, this trend was not strong enough to yield an overall main effect of orienting [orienting classification x trial ANOVA:

orienting classification x trial interaction, F(13,416) = 1.806, p=0.04; main effect of orienting F(1,32)= 3.661, p =0.065].

5.4.3.2. Conditioned Place Preference

Orienters and Nonorienters were subdivided into dosage groups, providing 4 groups for analysis: 1 mg/kg Orienter (n= 10), 1 mg/kg Nonorienter (n= 7), 2 mg/kg Orienter (n= 9), 2 mg/kg Nonorienter (n= 8). All groups showed place preference during both tests, as evidenced by paired t-tests comparing time spent in the drug-paired side during tests 1 and 2 to the time spent in that side during baseline testing (Figure 5.2). An orienting classification x dose x test (i.e. baseline, Test 1, test 2) repeated measures ANOVA of time spent in the drug-paired side revealed only a main effect of test, $F(2,60) = 52.25$, $p < 0.001$, indicating that during both Test 1 and Test 2 rats spent more time in the drug-paired side. Additional comparisons were made between dosage groups and orienting classification, and included an analysis of timing within the session (sessions were divided into 3 10-minute blocks). A dosage group x orienting classification x time block repeated measures ANOVA of time spent within the drug-paired side during Test 1 revealed a significant orienting classification x time interaction, $F(2, 60) = 5.08$, $p = 0.009$. Follow-up analyses revealed that for Orienters there was no effect of time ($p = 0.084$), while Nonorienters increased time spent in the drug-paired side as the session progressed, $F(2, 26) = 3.96$, $p = 0.031$. A dosage group x orienting classification x time block repeated measures ANOVA of time spent within the drug-paired side during Test 2 revealed no group differences in time spent within the drug-paired side.

In addition to considering the total time spent in both sides of the CPP chamber, we also compared the number of times rats crossed between sides (Figure 5.3). A dosage group x orienting classification x time block repeated measures ANOVAs revealed that

during both tests all groups crossed most frequently within the first 10 minutes of the session, and crossing decreased over time (Test 1 main effect of block, $F(2, 60) = 21.26$, $p < 0.001$; Test 2 main effect of block, $F(2, 60 = 93.65$, $p < 0.001$). Additionally, during Test 2 Nonorienters crossed more frequently than Orienters throughout the session, as revealed by a main effect of orienting classification, $F(1, 30) = 5.40$, $p = 0.027$. Follow-up analyses revealed that this effect was driven by Nonorienters in the 2 mg/kg dosage group. A repeated measures ANOVA of the crossing of Orienters and Nonorienters in the 1 mg/kg group revealed no effect of orienting classification ($p = 0.98$). However, for rats in the 2 mg/kg group, Nonorienters crossed more frequently than Orienters, $F(2, 15) = 12.30$, $p = 0.003$.

5.4.3.3. Ultrasonic vocalization

During all the conditioning days, rats emitted more FM calls than flat calls. We show that across all groups and across conditioning days, 8% of total USVs were flat type. The highest frequency of flat calls occurred on saline day 1, and the percentage decreased every day. An orienting classification x dose x day repeated measures ANOVA showed a main effect of day, $F(3, 60) = 3.83$, $p = 0.014$. During Tests 1 & 2, flat calls were also rare; during both tests flats comprised only 9% and 3% of total calls, respectively.

All groups called more frequently during Test 1 than during Test 2. Additionally, during Test 1 the majority of calls were made within the saline-paired side of the chamber (Figure 5.4), while during Test 2 the majority of calls were made within the drug-paired side (Figure 5.5). These findings were supported by an orienting classification x dose x test day x chamber side (drug-paired vs saline-paired) repeated

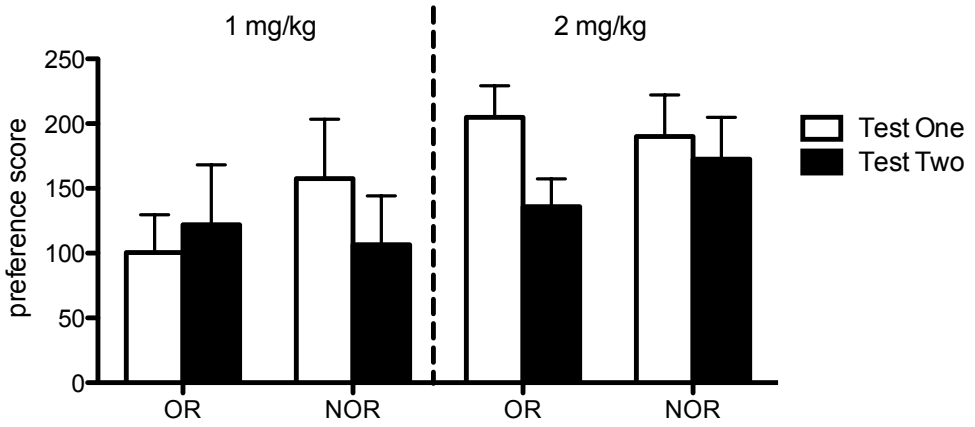
measures ANOVA showing a main effect of test day, $F(1, 30) = 4.83$, $p = 0.036$, and a test day x side interaction, $F(1, 30) = 8.87$, $p = 0.006$.

Orienters and Nonorienters in the 1 mg/kg dosage group did not differ in the number of calls made either in the drug-paired or saline-paired sides of the chamber (p's >0.1). However, for rats in the 2 mg/kg group, while in the drug-paired side Orienters made significantly more FM calls than Nonorienters ($p = 0.02$). An orienting classification x time block repeated measures ANOVA analyzing the number of FM USVs during Test 2 revealed that Orienters made more calls that Nonorienters, $F(1, 15) = 6.34$, $p = 0.023$, and that frequency of calling varied over time (peaking in the second time block – 10-20 minutes), $F(2, 30) = 3.62$, $p = 0.04$.

5.4.3.4. Correlation analyses

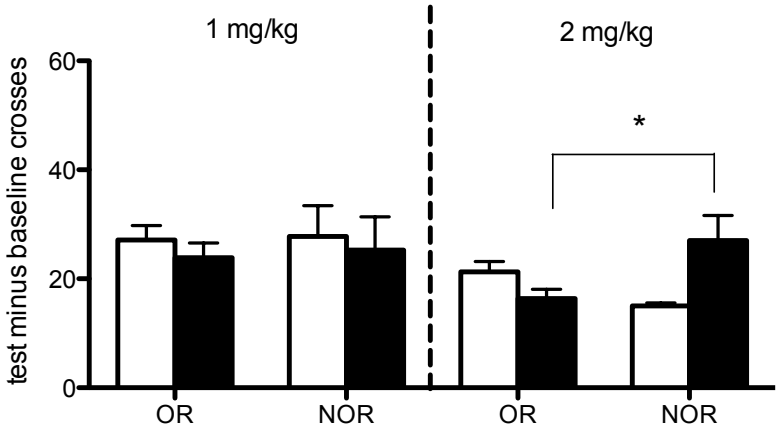
In addition to making group comparisons, we also analyzed our data for any correlations. A number of significant correlations are displayed in Table 5.1, but a few are of particular interest. Firstly, the number of vocalizations rats made during the first saline day predicted vocalization during the following training days as well as the first test session. Perhaps more interesting, heightened calling during saline day 1 also predicted a decrease in CPP during Test 2, i.e. rats that called more during saline day 1 did not show as large an increase in time spent in the drug-paired side from baseline to test 2. Also, rats that emitted more FM USVs during test 1 and test 2 also showed stronger CPP during test 2. Higher orienting scores predicted increased calling during saline training days as well as increased side-to-side crossing during test two.

Figure 5.2. Preference for the drug-paired context



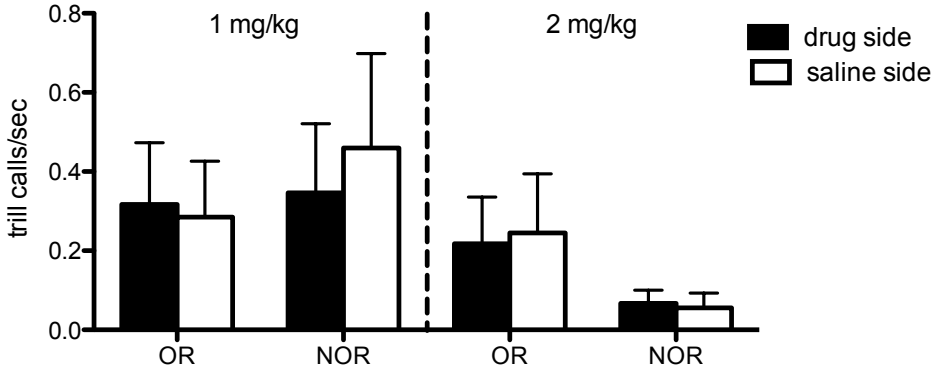
Mean (±SEM) increase in time spent in the drug paired side from baseline to Test 1 (white bars) and Test 2 (black bars). All groups showed an increase in time spent in the drug-paired side during both tests.

Figure 5.3. Crossing between sides during Tests 1 & 2



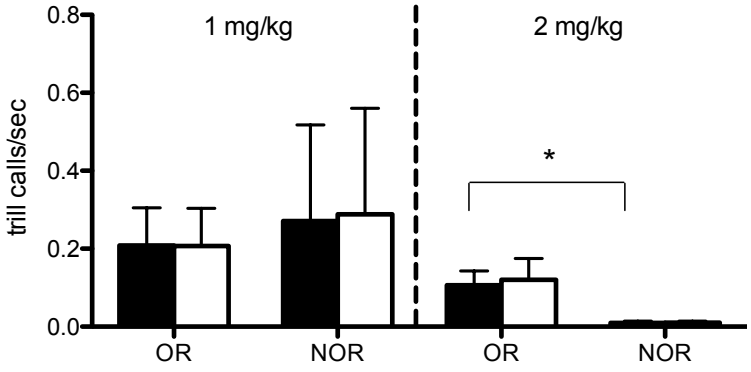
Mean (±SEM) increase in crossing from baseline to Test 1 (white bars) and Test 2 (black bars). In the 2 mg/kg dosage group, Nonorienters crossed more frequently that Orienters during Test 2.

Figure 5.4. Vocalizations within the drug and saline contexts: Test One



Mean (±SEM) number of FM vocalizations within the drug- and saline-paired contexts during Test 1.

Figure 5.5. Vocalizations within the drug and saline contexts: Test Two



Mean (±SEM) number of FM vocalizations within the drug- and saline-paired contexts during Test 2. For the 2 mg/kg dosage group, Orienters showed as increased rate of calling while in the drug-paired context, as compared to Nonorienters.

Table 5.1. Vocalization and preference correlational analysis

	1	2	3	4	5	6	7	8
1. Orienting score	-							
2. Saline day 1 FM	0.51**	-						
3. Saline day 2 FM	.40*	0.64**	-					
4. Amph day 1 FM	0.33	0.58**	0.80**	-				
5. Amph day 2 FM	0.29	0.52**	0.84**	0.894**	-			
6. Test 1 preference	-0.11	-0.27	0.08	0.03	0.01	-		
7. Test 2 preference	-0.03	-0.46**	-0.41*	-0.34*	-0.33	0.55**	-	
8. Test 1 crosses	0.17	-0.07	0.05	0.04	-0.13	-0.26	0.05	-
9. Test 2 crosses	-0.43*	0.01	-0.10	-0.07	-0.18	-0.22	-0.18	0.31

* significant correlations $p < 0.05$

** significant correlations $p < 0.01$

5.5. DISCUSSION

The current studies show a relationship between cue-directed responding and subjective effects of amphetamine. The studies here confirm previous reports that rats increase vocalization after saline injection (Knutson et al., 1999; Thompson et al., 2006), that amphetamine increases rates of ultrasonic vocalizations specifically by enhancing FM calls and suppressing flat calls, and also that rats emit 50-kHz calls in contexts that have previously been paired with reward (Knutson et al., 1998, 1999).

Ultrasonic vocalization findings from experiments 1 and 2 may appear to be in conflict given that Orienters called more than Nonorienters after amphetamine injection in experiment 1 but no differences existed after administration in experiment 2. However, previous work has indicated that the location of recording influences the rate of calling, with vocalizations being more frequent in the home cage than in a novel environment (Ahrens et al., 2013). Thus our finding that Orienters called more than Nonorienters when administered amphetamine in the home cage but that no differences existed in vocalizations within the CPP chamber are not conflicting and are, in fact, in line with the current literature.

Results from the human drug use/abuse literature show that there is individual variation in how enjoyable subjects find drug experiences, how positively they respond to drug-paired cues, and that these differences are predictive of propensity for addiction and relapse (De Wit and Phillips; 2012). While the current studies were not designed to assess drug-seeking and relapse per se, it is interesting that Orienters vocalized more when taking amphetamine in a familiar and safe environment (their home cage) and while in the amphetamine-paired context 12 days after their last amphetamine injection. This sustained enhancement of calling could be an indicator of stronger drug craving/expectation or perhaps indicate a stronger or more positive drug-memory elicited

by the context. However, as Orienters and Nonorienters did not differ in the amount of time spent within the drug-paired context when tested, this judgement cannot be made.

Given that both Orienters and Nonorienters showed a preference for the amphetamine-paired context and did not vary significantly in the time spent within that context, it is hard to make an argument for phenotypic differences in either their sensitivity to context conditioning or drug-seeking following place preference conditioning with amphetamine. However, correlational analyses indicated that higher orienting scores were predictive of enhanced vocalizations after saline injection, while enhanced saline vocalization predicted more vocalizations following amphetamine injection as well as a decreased preference for the drug-paired context when tested 12 days after conditioning. Previous reports have suggested that rats that exhibit high rates of calling after dopamine agonist exposure spend more time in the drug-paired side of a CPP chamber than rats that call less frequently (Ahrens et al., 2013; Meyer et al., 2012). The current study failed to show a relationship between calling and preference for a drug-paired context. All subjects here showed such strong preference for the drug-paired side, it seems plausible that this would mask any potential group differences. Perhaps the dosages used in the current study (1 and 2 mg/kg) were sufficient to induce place preference in all subjects and a lower dose might be more appropriate to detect subtle differences in dopamine sensitivity. Conversely, it might also be possible that using a higher dose of amphetamine would be aversive to some rats, allowing for greater variation in behavior.

It should be noted that the assertion that 50-kHz vocalizations are indicators of positive affect or hedonia is believed by some to be too simplistic. In fact, ultrasonic vocalizations are not readily induced by 3,4-methylenedioxymethamphetamine (MDMA), morphine, nicotine, or alcohol exposure, all drugs known to produce euphoria

and hedonic pleasure (Simola et al., 2012; Wright et al., 2012; Wiley and Spear, 2014). As the in depth study of ultrasonic vocalizations has only recently been made possible, much variation in the classification and interpretation of vocalizations exists and the possibility that 50-kHz calls are indicating something more complex that a rat's enjoyable drug experience should not be ignored. Given the relationships between cue-directed responding, dopamine sensitivity, place preferences, and vocalization reported here and elsewhere, it is clear that further investigation is warranted.

Chapter 6: Fos expression of Orienters and Nonorienters following amphetamine administration

6.1. ABSTRACT

When neutral cues are paired with reward, those cues can elicit approach and engagement. In the case of light-cues predictive of food reward, illumination of the light elicits conditioned orienting/rearing toward the light. While the orienting response (OR) habituates in some rats (Nonorienters) some continue to rear throughout training (Orienters). The OR is known to dependent upon dopaminergic amygdalo-nigrostriatal circuitry indicating that differences in the display of OR may be dependent upon variation in the functioning of this circuitry (El-Amamy & Holland, 2006; Gallagher et al., 1990; Han et al., 1997; Lee et al., 2005). The current study investigated Orienters' and Nonorienters' neural activation following dopamine agonist challenge, finding that both groups showed enhanced neural activation in the caudoputamen, substantia nigra pars reticulata, lateral portion of the amygdala central nucleus, and lateral habenula following amphetamine administration.

6.2. INTRODUCTION

When cues are paired with biologically significant events they can elicit conditioned responses. In the case of light-food pairings, two responses are readily observed: conditioned orienting to the light cue and conditioned approach toward the site of food delivery. The neural circuitry underlying conditioned orienting has been well characterized, and involves the central nucleus of the amygdala (CeA), dorsolateral striatum (DLS), and substantia nigra pars compacta (SNc) (El-Amamy & Holland, 2006; Gallagher et al., 1990; Han et al., 1997; Lee et al., 2005).

Previous evidence from this lab has shown that rats differ in their proclivity for the display of conditioned orienting. Olshavsky et al. (2013a,b) reported that some rats

show robust OR throughout training (Orienters), while others do not (Nonorienters). Additionally, work from Chapter 3 of this dissertation indicates that Orienters and Nonorienters differ in their tendency for risky decision-making, delay discounting, and attentional distraction, behaviors known to be dependent upon the dopaminergic circuitry. Finally, evidence from Chapter 4 shows that Orienters and Nonorienters differ in their subjective response to amphetamine exposure.

These findings led to the hypothesis that inherent differences in the nigrostriatal and mesolimbic dopaminergic circuitry underlie the array of behavioral differences in Orienters and Nonorienters. In order to investigate these potential differences in dopamine function, we examined expression of the immediate early gene product, Fos, after dopamine agonist challenge. Regions of interest were selected either for their role in conditioned cue-approach behavior, risky/impulsive decision-making, and/or reward processing and included the striatum (caudoputamen, nucleus accumbens core and shell) regions repeatedly shown to be necessary for conditioned orienting and other forms of conditioned cue approach, as well as risky and impulsive decisions (Han et al., 1997; Simon et al., 2011; Bechara, 2001; Goldstein et al., 2009; Rogers et al., 1999; Volkow et al., 2004); the habenula (medial and lateral divisions), a region recently shown to be necessary for some types of conditioned cue-approach (Danna et al., 2013); prefrontal cortical regions (orbitofrontal, infralimbic, and prelimbic cortices) known to be important for impulse control (Simon et al., 2011); substantia nigra (pars reticulata and compacta) and ventral tegmental area, necessary for conditioned orienting and reward learning (El-Amamy and Holland, 2006; Han et al., 1997; Lee et al., 2011); amygdala (central nucleus medial and lateral divisions, lateral amygdala, and basal amygdala) necessary for the conditioned orienting response and formation of cue-reward association (Gallagher et al., 1990; Han et al, 1997; Lee et al, 2005; El-Amamy and Holland, 2006).

6.3. MATERIALS AND METHODS

6.3.1. Subjects

Adult male Long-Evans rats (n=31, bred at UT-Austin) were double-housed in a reverse 12 h light/ 12 h dark cycle; lights off at 10 am. Rats had previously been fear conditioned with tone-shock pairings and extinguished. Rats were restricted to 90% free-feeding body weight, and this weight was maintained throughout Pavlovian conditioning procedures. Water was available ad libitum. All experiments were conducted according to the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

6.3.2. Experimental Design

6.3.2.1. Behavioral procedure

In order to classify rats as Orienters and Nonorienters, they were first training with Pavlovian light-food pairings. Pavlovian conditioning took place in eight identical conditioning chambers with aluminum sidewalls and ceilings and acrylic front and back walls (30.5 cm W x 25.4 cm D x 30.5 cm H, Coulbourn Instruments). The floor of the chamber consisted of stainless steel rods (0.5 cm diameter) spaced 1.0 cm apart. The food cup was mounted within the right wall of the chamber, 2.5 cm above the floor. Nosepokes to the food cup were detected by an infrared beam at its opening. A 2-W white light was mounted 20 cm above the foodcup. Illumination of the light served as a CS signaling grain pellet delivery to the foodcup. The left wall was concave and had five inactive ports. Each chamber was enclosed in a light- and sound-attenuated box (58.4 cm

x 61 cm x 45.7 cm); a ventilation fan provided masking noise. Digital cameras were mounted within each box and images were recorded during training.

In the initial training sessions rats simply learned to retrieve pellets from the food cup. Thirty pellets were delivered (variable ITI average 60 s) over a 30-minute session. After this session all rats reliably retrieved pellets from the food cup. The second session consisted of two parts. First, rats received 8 trials in which the stimulus light was illuminated but no grain pellets were delivered. These trials were meant to habituate the unconditioned rearing response that occurs to a novel light stimulus. For trials 9-16, a 10-s CS illumination co-terminated with grain pellet delivery to the foodcup. For the next 3 days of conditioning sessions consisted of 16 light-food pairings (variable ITI averaging 120 s). Twenty-four hours after this session rats were either given 20 g of grain pellets in a clean cage or placed in a clean cage for 30 minutes. Rats were then returned to the training chambers for 18 light-only trials (data not reported here). After this session rats were returned to ad libitum feeding.

Three to ten days after the final training session rats were administered 2 mg/kg d-amphetamine (n=16) or saline (n=15) via intraperitoneal injection and placed in their homecage before being sacrificed.

6.3.2.2. Histology

Ninety minutes after injection rats were overdosed on Euthasol (TW Medical, Denver, CO) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and stored in 4% paraformaldehyde/20% sucrose solution and refrigerated overnight. Twenty-four hours after perfusion brains were frozen in powdered dry ice and stored at -80° C. Brains were sliced on a freezing microtome; 30 μm coronal sections were collected in six series.

6.3.2.3. Immunohistochemistry

The first set of sections was mounted onto slides and Nissl stained. The second set was used for Fos immunoreactivity. Endogenous peroxidase was washed from free floating sections in 0.3% H_2O_2 in 0.1 M phosphate buffer (PB) containing 0.9% saline (PBS) for 30 minutes. After several rinses in PBS, the tissue was incubated for 1 hour in PBS with 3% normal goat serum (Vector Laboratories, Brulingame, CA) and 0.3% Triton X-100 (PBST). Sections were then incubated for 72 hours in rabbit *c-fos* antibody (diluation 1:1000, Santa Cruz Biotechnology) in PBST; incubation was held at 4 ° C. After this primary antibody incubation sections were washed in PBS and incubated for 1 hour in biotinylated goat anti-rabbit IGg (dilution 1:250, Vector Laboratories) in PBST. After several PBS rinses sections were finally incubated in avidin-biotin conjugate for 1 hour. After rinses tissue was color reacted with DAB (Sigma-Aldrich), mounted on slides, dehydrated in ascending concentrations of alcohol, defatted in xylene, and coverslipped with Permount.

6.3.2.4. Analysis of Fos expression

All analyses were conducted blind of experimental condition. Regions of interest within the nigrostriatal and mesocorticolimbic dopamine systems were selected and included the caudoputamen (dorsolateral and ventromedial divisions), nucleus accumbens (core and shell), habenula (medial and lateral), infralimbic, prelimbic and orbitofrontal cortices, substantia nigra (pars reticulata and pars compacta), ventral tegmental area, and amygdala (medial-central, lateral-central, lateral, and basal nuclei). Brain areas were defined according to Swanson's brain atlas (Swanson, 2003). For each region, 1-4 sample areas were defined. Cell bodies stained darker than 3 standard deviations beyond background threshold were autocounted using ImageJ software (Rasband, 1997-2001).

6.4. RESULTS

6.4.1. Pavlovian conditioning

As in previous experiments reported in this dissertation, there was an overall acquisition of both orienting and food cup approach behavior. Rats were classified as Orienters and Nonorienters based upon their average orienting score during the last 8 trials of training. Rats scoring at or above the median orienting score were classified as Orienters (n = 16), while those below were Nonorienters (n= 15). During these trials Orienters showed significantly more OR [orienting classification x trial repeated measures ANOVA, main effect of orienting $F(1, 29) = 46.31$, $p < 0.001$]. No differences existed in groups' food cup behavior ($p = 0.58$).

6.4.2. Fos expression

Fos-positive cells within sampled regions of interest of both hemispheres were counted and summed. Four regions showed a main effect of drug administration. The lateral habenula (LHb), substantia nigra pars reticulata (SNr), caudoputamen (CP), and amygdala central nucleus – lateral division (CeA-L) all showed a significant increase in Fos-positive cells for both Orienters and Nonorienters that received amphetamine injection (Figure 6.1). These findings were supported by orienting classification x drug/saline injection ANOVAs of Fos-positive cell counts [main effects of drug/saline injection: LHb $F(1, 26) = 15.99$, $p < 0.001$; SNr $F(1, 26) = 5.37$, $p < 0.05$; CP $F(1,26) = 7.86$, $p < 0.01$; CeA-L $F(1, 26) = 5.43$, $p < 0.05$].

More in-depth analyses considering the anterior-posterior (A – P) distribution of cells revealed a more complex pattern of expression. Regardless of amphetamine administration, the majority of Fos-positive cells were located within dorsal and anterior portions of the CP; this effect was stronger for Nonorienters than Orienters. Both groups

showed an increase in Fos expression following amphetamine administration; dorsal and anterior portions of the CP showed the greatest enhancement of Fos expression. These findings are supported by direction (dorsal/ventral) x A- P level x orienting classification x drug administration repeated ANOVAs of Fos-positive cells counts in the caudoputamen showing main effects of both direction, $F(1, 26) = 141.93$, $p < 0.001$, and level $F(2, 52) = 79.6$, $p < 0.001$, as well as a level x orienting interaction, $F(2, 52) = 3.85$, $p < 0.05$. Additionally, there was a main effect of amphetamine administration, $F(1, 26) = 6.92$, $p < 0.05$, and both level x drug, $F(2, 52) = 10.90$, $p < 0.001$, and direction x drug, $F(1, 26) = 25.03$, $p < 0.001$, interactions were significant.

In the LHb anterior portions showed higher Fos expression, although the effect was weaker for rats receiving an amphetamine injection: main effect of drug $F(1, 24) = 18.58$, $p < 0.001$; main effect of level $F(4, 96) = 13.11$, $p < 0.001$; level x drug interaction $F(4, 96) = 3.40$, $p < 0.05$.

Within the orbitofrontal cortex (OFC), Fos expression was most concentrated in the posterior levels. Orienters injected with both saline and amphetamine showed a significant increase in expression from anterior to posterior portions, as did Nonorienters injected with saline. Only Nonorienters injected with amphetamine failed to show a significant effect of A – P level. Main effect of level $F(2, 50) = 26.94$, $p < 0.001$; level x orienting x drug $F(2, 50) = 3.10$, $p = 0.054$; follow-up repeated measures ANOVA of each group separately: Orienters: saline $F(2, 12) = 8.27$, $p < 0.01$; Orienters: amphetamine $F(2, 14) = 8.16$, $p < 0.01$; Nonorienters: saline $F(2, 10) = 9.29$, $p < 0.05$; Nonorienters: amphetamine $F(2, 14) = 3.02$, $p = 0.08$.

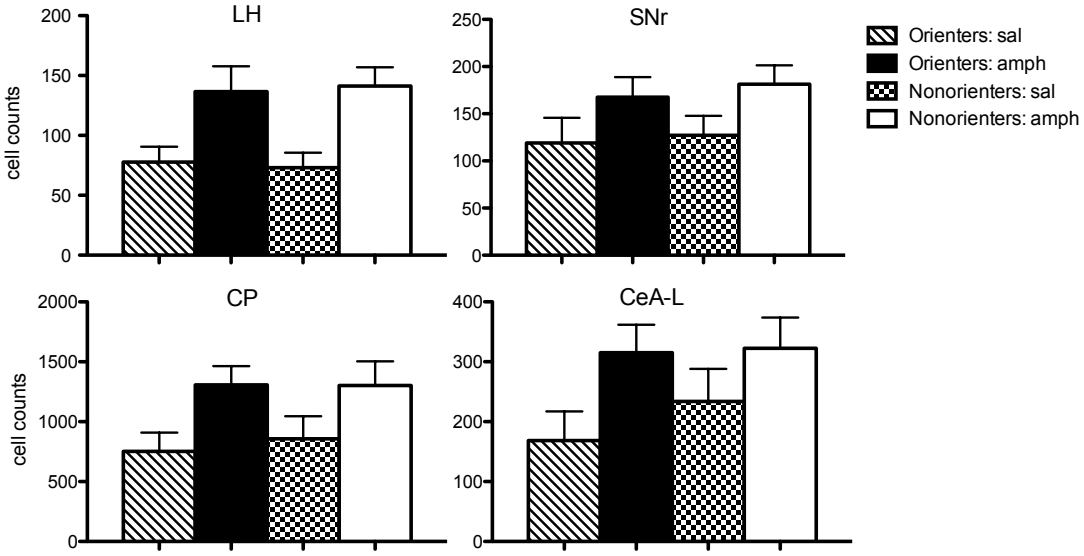
Orienters and Nonorienters showed enhanced Fos expression in SNr after amphetamine administration, and expression was highest in the posterior levels, main

effect of drug $F(1, 26) = 5.37$, $p < 0.05$ and main effect of level $F(4, 104) = 23.44$, $p<0.001$.

In the CeA-L, Fos expression increased moving anterior to posterior, and this effect was strongest for rats that received an amphetamine injection, level x drug interaction $F(3, 78) = 4.09$, $p < 0.01$; main effect of level $F(3, 78) = 18.03$, $p < 0.001$; main effect of drug $F(1, 26) = 5.43$, $p < 0.05$.

Three regions, the lateral amygdala (LA), basal amygdala (BA), and ventral tegmental area (VTA) showed only an effect of A – P level. In the LA, Fos-positive cells were concentrated in more posterior portions, main effect of level $F(4, 104) = 8.98$, $p < 0.001$. In both the BA and VTA, cells counts were higher in anterior portions; BA main effect of level $F(6, 156) = 7.16$, $p < 0.001$; VTA main effect of level, $F(3, 78) = 29.11$, $p < 0.001$.

Figure 6.1. Fos expression of regions showing effect of amphetamine administration



Mean (±SEM) number of cells counted within each region showing a main effect of amphetmine administration. Note the differing ranges of the y axes.

6.5. DISCUSSION

The current study sought to characterize underlying differences in the dopaminergic function of Orienters and Nonorienters, but here we report that Orienters and Nonorienters show comparable patterns of neural activation following amphetamine administration. Both groups showing enhanced Fos expression in the LHb, CP, SNr, and also the CeA-L after amphetamine injection. It should be noted that while Fos expression cannot be considered a direct reflection of dopaminergic activation within these regions, the LHb, CP, SNr, and CeA-L are regions all known for there robust dopaminergic innervation.

It has long been reported that the LHb exerts inhibitory control over the substantia nigra and ventral tegmental area and due to its influence over these regions, interest has recently piqued regarding its role in learning and the attribution of cue salience (Christoph et al., 1986; Friedman et al., 2010, 2011; Ji and Shepard, 2007; Matsumoto and Hikosaka, 2007). Danna et al. (2013) reported that rats with LHb lesion show enhanced cue approach, and Flagel et al. (2011) reported that rats prone to cue-directed responding showed enhanced neural activation in the LHb following cue exposure. Results from Flagel et al. (2011) could be attributed to underlying differences in the LHb function of cue-approachers and non-cue-approachers or a difference in neural activation that arises after cue exposure. While the modality of the CSs in these reports is different (a light CS vs lever CS) our findings suggest that the differences in neural activation reported by Flagel et al. (2011) are not explained by baseline differences in the function of the LHb, but that these differences arise after exposure to reward-paired cues.

Orienters and Nonorienters both showed an increase in striatal activation following amphetamine injection. All addictive drugs have the ability to increase dopamine in the striatum, and it is believed that the effect underlies the user's feeling of

subjective reward (Koob, 1992). There is also ample evidence that drugs abusers show degeneration in the striatum, even after long periods of abstinence (McCann et al., 1998; Sekine et al., 2001; Volkow et al., 2001). While many studies have investigated the role of the ventral striatum (nucleus accumbens) in drug seeking and drug use, recent evidence has highlighted a role for the dorsal striatum. Amphetamine users with dampened activation of the dorsal striatum were more likely to relapse and drug-seeking behaviors in addiction-vulnerable individuals were correlated with increases in dopamine within the dorsal striatum (Belin and Everitt, 2008). The results reported here add to mounting evidence that the dorsal striatum is an important target of amphetamine action.

An increase in activity within the SNr of amphetamine-injected rats is not surprising given the numerous reports that release of dopamine from these neurons is responsible for amphetamine's locomotion-enhancing effects (Jackson and Kelly, 1983, 1984; Stewart & Vezina, 1989; Timmerman and Abercrombie, 1996). Moreover, this region receives massive afferent projections from the striatum, particularly the dorsal portion, a region that also showed an effect of amphetamine administration. While the dopamine circuitry as a whole changes after repeated drug use, recent evidence from Granado et al. (2010) and Wang et al. (2004) suggests that this nigrostriatal pathway may be the pathway most vulnerable to destruction.

The central nucleus of the amygdala has garnered some discussion within the drug addiction literature due to its role in the autonomic and behavioral responses to stress and anxiety (Karanikas et al., 2013). Much of the work regarding the role of this region in drug use/abuse has focused on corticotropin releasing factor (CRF) within the central amygdala, suggesting that changes to CRF-expressing cells may underlie many of the emotional and behavioral symptoms of addiction (Koob, 2003; Heilig and Koob, 2007; Gilpin and Roberto, 2012). Little is known of the direct projections to and from the CeA-

L, as its small size has precluded much tract tracer investigation (though see Petrovich and Swanson, 1997). Much of the discussion of this region has more broadly considered the amygdala central nucleus as a whole, including both medial and lateral divisions. As a unit the central nucleus is positioned to receive broad sensory inputs and gate motor responses (LeDoux et al., 1990a; Mascagni et al., 1993; McDonald and and Mascagni, 1996; Romanski and LeDoux, 1993; Rizvi et al., 1991; Schwaber et al., 1982). The work presented here suggests that the CeA lateral and medial divisions differ in their responses to drug exposure and that investigations into the separate functions of these regions are warranted.

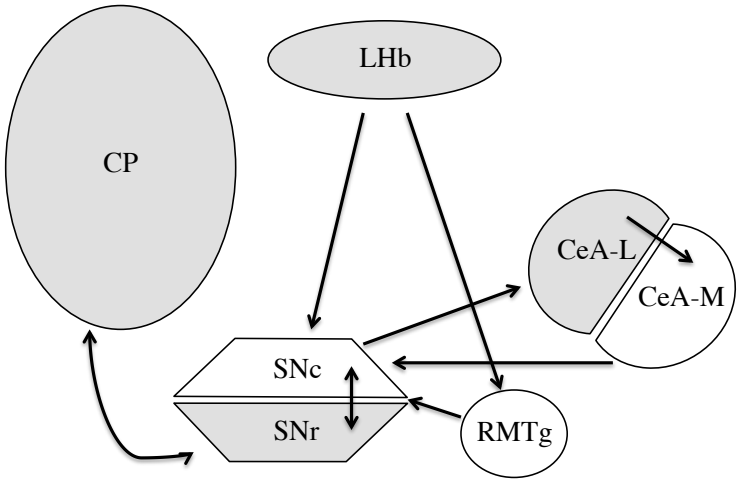
Finding significant drug effects within the LHb, CP, SNr, and CeA-L prompts the search for possible neural circuitry linking the four structures (Illustration 1). The LHb sends sparse projections to the SNc, which is highly interconnected with the SNr (Araki et al., 1988; Herkenham and Nauta, 1979; Lee and Tepper, 2009). However, recent evidence suggests that it the LHb is more likely to appoint its inhibitory control the substantia nigra via indirect projections mediated by the rostromedial tengmental nucleus (RMTg) (Jhou 2005; Jhou et al., 2009; Hikosaka et al., 2008). In contrast, the CP provides excitatory input the SNr, while the central amygdala provides input to SNc (Do et al., 2012; Gonzales and Chesselet, 1990; Haber and Knutson, 2010; Lee et al., 2005). Reciprocal connections between the two parts of the substantia nigra provide opportunity for the amygdala input to influence SNr (Lee and Tepper, 1990).

Because I hoped to find underlying differences in the functioning of the dopamine system, I chose to examine Fos expression not after exposure to a reward-paired cue or context, but after a dopamine challenge. As conditioned orienting is dependent upon circuitry of the amygdala central nucleus, dorsolateral striatum, and substantia nigra pars compacta, my prediction was that Orienters' robust orienting was the product of

enhanced function within these regions and expected to that Orienters would show stronger Fos expression in these regions, as compared to Nonorienters. However, none of the regions showed an effect of orienting classification. It is possible that a 2 mg/kg dose of amphetamine induced such strong activation within these regions that subtle differences in responsiveness to dopamine agonism were washed out.

While there were no significant group differences reported here, follow-up studies investigating dopamine receptor expression of Orienters and Nonorienters may be better equipped to detect differences in the brains of these groups. Given the robust behavioral differences in Orienters and Nonorienters, all of which can be related to the function of dopaminergic regions, it seems highly likely that neurochemical differences exist between the two groups and that the current study was not sensitive enough to detect them.

Illustration 1. Potential interactions between LHb, CP, SNr, and CeA-L



An illustration depicting possible mechanisms of interaction between the four regions showing an effect of amphetamine administration (in grey) and some closely related regions that may take part in indirect pathways of communication (in white).

Chapter 7: General Discussion

This dissertation describes the first work characterizing individual differences in the expression of cue-directed orienting during Pavlovian light-food pairings and supports the assertion that differences in subjects' propensity for cue-directed responses are associated with differences in behavior across a range of assays. In Chapter 2 I reported that Orienters and Nonorienters differ in their susceptibility to memory update, that Orienters are vulnerable to updating of appetitive memories via extinction within a reconsolidation window whereas Nonorienters' conditioned responding persists, and that this vulnerability is reliant upon the central nucleus of the amygdala. Chapter 3 investigated the role of orienting phenotype in fear conditioning and memory update, reporting that while extinction within a reconsolidation window worked to attenuate the spontaneous recovery of freezing for both Orienters and Nonorienters conditioned with a 1.0 mA shock, Orienters showed a more dramatic reduction in freezing to the CS. Nonorienters showed more context freezing.

In Chapter 4 I characterized the behavior of Orienters and Nonorienters in a variety of assays for impulsivity, risk-taking, and attentional processing, showing that Orienters are more apt to choose small immediate rewards over larger delayed ones, are more willing to risk foot shock to receive a larger reward, are quicker to exit a familiar enclosed environment for a novel open field, and are more vulnerable to visual distractions. Together these findings indicate that Orienters, as compared to Nonorienters, are more apt to make risky and impulsive decisions and are more easily distracted from tasks requiring sustained attention.

As conditioned orienting and propensity for impulsivity and risk-taking are dependent upon dopaminergic circuitry, Chapter 5 characterized Orienters' and Nonorienters' response to dopamine agonist exposure. Experiment 1 investigated 55-kHz ultrasonic vocalization following injection of amphetamine within rats' homecage, finding that Orienters vocalized more than Nonorienters and that this enhancement was driven by a dramatic increase in frequency-modulated calls. Experiment 2 reported that while Orienters and Nonorienters both prefer an amphetamine-paired context, Orienters vocalize more while in that context when tested, drug free. Finally, in Chapter 6, I investigated neural activation following amphetamine administration, reporting that both Orienters and Nonorienters showed an increase in Fos expression in the lateral habenula, substantia nigra pars reticulata, caudoputamen, and lateral portion of the amygdala central nucleus after a 2 mg/kg amphetamine injections.

All of the experiments described in this dissertation classified Orienters and Nonorienters under very specific conditions, and the patterns of orienting and food cup approach described here would be expected to change were the parameters of training changed. First, the interval between light-CS presentation and grain pellet-US was always 10 s. The topography and distribution of CR is dependent upon this interval, as longer CSs produce more robust CS-approach (Gibbon and Balsam, 1981). Also, localizability and spatial separation between the CS and US are important factors affecting the display of conditioned responses; enhanced localizability and decreased spatial separation between the CS and site of US delivery increases cue approach (Boakes, 1977; Holland, 1980). Additionally, all the light-food training procedures described here involved 56-pairing trials. Reporting persistent conditioned orienting after training with 56 light-food pairing trials is in line with the literature, as Chess et al. (2005) reports continued conditioned orienting after training with 60 light-food pairings.

It is unknown how over-training rats on a light-food pairing protocol would affect conditioned orienting, as the studies presented here only trained until food cup responding came to an asymptote.

Also of note, all the Pavlovian conditioning was done under circumstances of food deprivation, which, it has been suggested, creates a state of altered reward sensitivity (Carr, 2002; Frank et al., 2012). The food deprivation protocol used in the studies described here was not particularly dramatic, as rats were reduced to 90% their free-feeding weight. Additionally, Boakes (1977) reports that degree of food deprivation is less important to the elicitation of cue- and reinforcer-directed responses than other factors, such as reinforcement schedule, which may be manipulated to enhance cue or food cup approach (100% reinforcement enhances food cup approach as 50% reinforcement supports enhancement of cue-approach). Robust effects of food deprivation have been identified in studies of addictive behavior, with a number of studies showing that food restriction increases drug self-administration and lowers the threshold at which rats find drugs reinforcing (Carroll, France, and Meisch, 1979; Carroll & Meisch, 1984). Importantly, for the amphetamine administration and CPP experiments described here rats were not food deprived during the drug-administration portions of the experiment.

The possibility that prior experience with light-food pairings affected performance in the subsequent behavioral tasks is impossible to rule out, as the majority of experiments reported in this dissertation classified Orienter/Nonorienter phenotype prior to testing in other behavioral tasks. There is one exception: performance in the light-dark box emergence task in Chapter 3 was assessed prior to Orienter/Nonorienter classification. However, in Chapter 4 classification occurred before the light-dark box emergence assay. Importantly, similar patterns of results were reported regardless of

whether Pavlovian conditioning took place before or after light-dark box emergence tasks. Because the Pavlovian conditioning took place in separate contexts, even in separate laboratories, from the delay discounting, risky decision-making, light-dark box emergence, and drug exposure/conditioning tasks, differences in performance of these tasks cannot be attributed to previously learned context-dependent differences in motivation or arousal.

Rather than suggest that the OR per se is the important focus of the work described in this dissertation, I would propose that the two phenotypes, Orienters and Nonorienters, have preexisting differences in neural circuitry and that persistent or habituating conditioned OR is a side effect of this difference. Circumstantial evidence regarding the role of amygdalo-nigrostriatal dopamine in conditioned OR as well as the role of these regions in the behavioral assessments reported here (delay discounting, risk-taking, attention) suggests that this circuitry may be functioning differently in the two phenotypes. This hypothesis is supported by other work reporting that individual differences in conditioned cue approach are heritable (Fitzpatrick et al, 2013; Flagel et al., 2010). However, the possibility exists that underlying differences in the neural function of Orienters and Nonorienters are exacerbated by training with light-food pairings and that this influences later performance. Previous studies report that as light-food training progresses, conditioned OR's reliance upon CeA-SNc circuitry shifts to reliance upon striatal-SNc as OR is acquired and become habitual (Han et al., 1997; McDannald et al, 2004). It is possible that Orienters make this shift more readily, behaviorally reflected by their persistent OR, and that this shift initiates lasting changes in striatal-SNc function that influence performance in subsequent tasks.

Orienters could have a sensitized dopamine circuitry, as compared to Nonorienters, but the reverse could also be true: that Nonorienters have heightened

dopaminergic function. Differences in dopamine sensitivity could be driven by phenotypic differences in dopamine synthesis, release, or receptor density. Humans diagnosed with Attention Deficit/Hyperactivity Disorder (ADHD), a diagnosis characterized by impulsivity and inattention, exhibit marked abnormalities in cortical dopamine receptor density, dopamine neurotransmission, and striato-cortical, ventral tegmental area, and substantia nigra connectivity (Jucaite et al., 2010; Swanson et al., 2000; Tomasi and Volkow, 2012, 2014). Orienters share some characteristics with humans diagnosed with ADHD, namely distrability when presented with extraneous stimuli (Orienters are more likely to be distracted by a visual distractor in the 5-CSRTT) and impulsivity (Orienters are less willing to wait for reward and venture more readily into a novel open field) (American Psychiatric Association, 2013). Identification of Orienter and Nonorienter phenotypes and their potential differences in dopamine function provide the opportunity for investigation of potential environmental and genetic factors which impact development and dopamine function in adulthood. While ADHD is present in only 2-5% of the population and Orienters, due to methodological choices comprised 50% of the samples studied here. The study of Orienters and Nonorienters may serve to characterize the range of "normal" function and make distinctions about when and how function becomes pathological.

In addition to the potential utility of Orienter/Nonorienter classification in characterizing individual variation in dopamine function, the work described in this dissertation serves to account for some of the behavioral variability that exists in randomly-bred unaltered Long-Evans rats, directly purchased from a breeding facility. Considering both the work in this dissertation and other unpublished data, I have classified over 500 rats from three different breeders (Harlan, Long-Evans, UT-Austin), of two different strains (Long-Evans and Sprague-Dawley), and both sexes, consistently

finding robust variation in rats' propensity for conditioned OR. While I have made distinctions here between Orienters and Nonorienters, presumably rats of both phenotypes comprise typical experimental and control groups. The inclusion of both phenotypes, shown in this dissertation to exhibit robust behavioral differences, serves to increase within-group variation. Increases in error variance reduce both sensitivity and power to detect between-group differences, and accounting for some of this variability increases the likelihood of finding significant group differences. This point is best illustrated by the findings presented in Chapters 2 and 3. For the experiments reported in Chapter 2, initial efforts to identify a main effect of retrieval + extinction failed and variability in food cup approach during the test for spontaneous recovery was high. Rats that received a retrieval trial prior to extinction showed a range in food cup responding during the spontaneous recovery test, with individuals nosepoking anywhere from 0 to 10 times during the 4 trials. Upon classification of individuals based upon conditioned OR behavior, group variability was reduced and an interaction effect was detected. In Chapter 3, the freezing-attenuating effect of retrieval + extinction was robust enough to be detected regardless of Orienter/Nonorienter classification. However, while receiving a retrieval trial prior to extinction prevented a significant increase in freezing from extinction to spontaneous recovery test for both phenotypes, a range in freezing was apparent during this test. This variation in freezing could be partially accounted for by classification as an Orienter or Nonorienter.

While I am the first to characterize rats based on light-cue-directed conditioned orienting phenotypes, other researchers have simultaneously been characterizing rats prone to approach and engage with a lever cue predictive of food reward (Flagel et al., 2011; Meyer et al., 2012; Morrow et al., 2011; Robinson and Flagel, 2010; Saunders et al., 2013; Yager et al., 2011). In fact, some of the behavioral findings discussed in this

dissertation, e.g. increased impulsivity and subjective response to drugs of abuse, have been investigated in these lever-approaching rats (Lovic et al., 2012; Meyer et al., 2013). While the current dissertation work and extensive research from the lab of Terry Robinson both describe rats prone to cue-directed and reward-directed responses, important distinction exists. Importantly, lever-approachers engage exclusively with a lever-cue predictive of reward, precluding their approach toward the site of reward delivery. However, Orienters show both cue- and reward-directed behaviors during CS presentation. In addition to noting these differences, cue modality should also be considered. In the case of a lever-CS, the cue can be engaged with and manipulated, whereas in the experiments described here light-CSs were only observed. Recent evidence from Chang and Holland (2013) suggests that the neural circuitry underlying conditioned cue approach differs depending upon modality of the cue and that the processing of simple visual cues differs from processing of cues that can be engaged with.

Together, the findings presented in this dissertation suggest that propensity for light cue approach is predictive of behavioral response to a variety of assays. Orienters are more likely to make risky and impulsive decisions, enter novel environments, and show enhanced subjective response to amphetamine administration. Given the evidence that humans prone to drug addiction frequently display these same characteristics, future studies investigating potential differences in Orienters and Nonorienters self-administration behaviors would be interesting. Historically, preclinical drug research has ignored individual differences, even though it is common knowledge that some people try drugs and become addicts and some use them only a few times. Some effort has been made to breed for addiction-like characteristics, e.g. high novelty-seeking, and then look for neurochemical and behavioral differences during drug use; however, this approach

may not be ideal as it reduces the complex issue of drug abuse susceptibility to a single trait (Wooters and Bardo, 2011; Tournier et al., 2013). It may be more fruitful then to use the phenotypes described here to characterize the development of these traits and explore the relationships between behavior, neurochemistry, and susceptibility to abuse. Moreover, while results from Chapter 2 address Orienters' and Nonorienters' response extinction after training with a food reward, they may speak the phenotypes' larger differences in the ability to extinguish responding to reward-paired cues. While Orienters' behavioral traits may suggest an enhanced vulnerability to drug use/abuse, they may also be more responsive to behavioral interventions aimed at curbing consummatory responses.

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