The Neural Substrates of Specific and General Aversive Motivation

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May 2016

A thesis in the Department of Psychology submitted to the faculty of the Graduate School of Arts & Science in partial fulfillment of the requirements for the degree of Master of Arts at New York University

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Abstract

In contrast to appetitive studies, motivation in the aversive domain has not been given much attention. Utilizing the Pavlovian to instrumental transfer (PIT) paradigm, where a Pavlovian conditioned stimulus (CS) that has been paired with an unconditioned stimulus (US) facilitates instrumental avoidance responses, this study examined the neural basis of how Pavlovian signals can invigorate instrumental responses under two distinct conditions. CS1 (e.g., tone) is a threat signifying oncoming shock while CS2 (e.g., noise) predicts a klaxon. Both CSs are capable of elevating ongoing shock-avoidance behavior rates, but through distinct psychological processes (i.e., sensory-specific versus sensory-general). Using chemogenetics, we inhibited neural activity in the basal (BA) or central (CeA) amygdala during tests for these different forms of motivation. BA inactivation reduced specific (i.e., shock) but not general (i.e., klaxon) motivation. Inhibition of central amygdala reduced both specific and general motivation. These data suggest that threat processing in the aversive domain may depend on internal representations that do not conform to what has been found in appetitive studies.

Keywords: aversive learning, aversive motivation, Pavlovian-to-instrumental transfer (PIT), amygdala, DREADD, KORD, rat

The Neural Substrates of Specific and General Aversive Motivation

When organisms suddenly face danger, their typical defensive *reactions* include freezing, fleeing, and fighting (Lorenz & Tinbergen, 1938; Tinbergen, 1951). These automatic responses to threat may have developed to aid survival, but they are not the only options available. Humans and animals can learn various *actions* through experience where they find that certain behaviors lead to their obtaining a goal or reward (Skinner, 1938; Estes & Skinner, 1941; Estes, 1948). These behaviors are known as instrumental actions, and they are active responses to threats that often provide better outcomes than reactive responses like freezing, which in the face of a speeding car or in the middle of a battlefield can prove ineffective.

Studying how actions work in an experimental setting first requires an understanding of what constitute and drive actions. A comparison between reactions and actions provides a helpful contrast that illustrates the qualities in actions. Reactions are not learned behavior; they are innate, inflexible, and are evoked by stimuli in the environment (Campese, Sears, Moscarello, Diaz-Mataix, Cain, & LeDoux, 2015). Increased heart rate (Vrana, Cuthbert, & Lang, 1989; Lang, McTeague, & Bradley, 2016) and freezing on the spot are reactions in the face of terror as one becomes surrounded by a swarm of bees. On the other hand, actions are learned, flexible, and involves more than just a stimulus (Skinner, 1938; Estes & Skinner, 1941; Estes, 1948; Niv, Joel, & Dayan, 2006). Jumping into the river away from a swarm of angry bees is an action. Thus, many things contribute to actions: previous experience, the internal state (including arousal, motivation, etc.), and what goals the animal aims to achieve (Campese et al., 2015).

The main task and paradigm of this study, Pavlovian-to-instrumental transfer (PIT), is not only able to segregate reactions from actions, but also provides a way to examine how threats can modulate or motivate an animal's actions (Campese, McCue, Lázaro-Muñoz, LeDoux, &

Cain, 2013; Campese, Kim, Lázaro-Muñoz, Pena, LeDoux, & Cain, 2014; Moscarello & LeDoux, 2014). In other words, the PIT paradigm offers a behavioral model of how rats and humans can concurrently track and respond to multiple threats (Nadler, Delgado, & Delamater, 2011; Campese et al., 2013).

Overview of the Pavlovian to Instrumental Transfer Paradigm

The Pavlovian to instrumental task integrates Pavlovian (or classical) conditioning with instrumental conditioning to examine how the former affects the latter. PIT studies are normally comprised of three phases: Pavlovian conditioning, instrumental conditioning, and PIT testing. The first two phases are well established – they are the two main paradigms used to study learning processes. Early experimental investigations on learning came into prominence with Thorndike (1898), Pavlov (1927), Watson (1929), and Skinner (1938). Pavlov and Watson propounded classical conditioning, and Thorndike and Skinner became the main proponents of instrumental conditioning (for more on individual contributions see Bouton, 2007). The following two subsections cover the tasks involved in each paradigm with a focus on the aversive domain.

Pavlovian conditioning. One of the most well-known experiments in psychology is Pavlov's (1927) conditioning of dogs to salivate when they hear a bell ringing. Pavlov's experiments demonstrated that animals can come to associate previously unrelated stimulus like a bell ringing with a biologically salient event like food. His training involved exposing dogs to a conditioned stimulus (CS) like a bell ring before presenting them with an unconditioned stimulus (US) like food. Before pairing the bell ring CS with the food US, there was no reason for the dogs to salivate to the bell ring. However, this CS-US pairing led to dogs salivating when the bell ring CS was presented. Thus, salivation, which was originally an unconditioned response

(UR) to food, became a conditioned response (CR) to the bell ring. This finding was so influential that it became known as Pavlovian or classical conditioning.

Classical conditioning extends beyond *appetitive* (utilizing pleasant USs like food) learning. *Aversive* (involving unpleasant USs like electric footshock) classical conditioning procedures use CSs like auditory tones and noise or visual stimuli like blinking lights, and measure things like freezing behaviors and eye blink responses as CRs (Pavlov, 1929; Blanchard & Blanchard, 1969; Bolles & Fanselow, 1980). Most of the investigations on aversive learning use Pavlovian threat conditioning (PTC) procedures like the one shown in Figure 1. This is due to the powerful replicability of the PTC paradigm that has proved consistently that one time learning of the CS-US (e.g., tone-shock) association lasts for even years in animals (Gale et al., 2004). PTC allows the animal to interpret the CS as a threat, leading the CS to induce automatic defensive reactions that are normally elicited by a US (LeDoux, 2000).

Because classical conditioning involves the animals learning to associate different stimuli in their environments, this paradigm can be likened to stimulus learning (Bouton, 2007). The responses in classical conditioning are reactive in nature which provide a way for experimenters to investigate reactions to threats.

Instrumental conditioning. In contrast to classical conditioning that focuses on stimulus learning and reactions, instrumental or operant conditioning offers ways to study how animals learn about the relationship between their actions and consequences. Thorndike's (1911) law of effect summarizes the framework in which animals learn to do things that would either benefit them or lead to bad consequences. There are four types of learning: reward learning, punishment learning, omission learning, and avoidance learning. Reward learning is when the animal's behavior produces a biologically beneficial event (e.g. a dog is told to sit and when she does, she

gets a treat). Punishment learning involves the animal's actions leading to a bad or painful event (e.g. dog running around in the house with muddy feet gets scolded). Compared to reward and punishment learning where the animal's action *produces* biologically positive or negative events, omission and avoidance learning results in the animal's behaviors *preventing* good or harmful events. While omission learning is about preventing benefits (e.g. dog not barking when an intruder comes in leads to no treat), avoidance learning involves an organism's action preventing negative experiences (e.g. a dog not chasing after a cat across the street full of cars keeps the dog from being hit by cars).

Conditioning tasks were used to study avoidance learning throughout the twentieth century (Miller 1948, 1951; Mowrer 1947; Mowrer & Lamoreaux, 1946; Campese et al., 2015). Avoidance conditioning can be divided into active avoidance and passive avoidance. Active avoidance is where animals learn to perform actions to avoid harm, whereas passive avoidance is when animals learn to withhold actions to avoid harm (Campese et al., 2015). More specifically, as the unsignaled Sidman active avoidance (USAA) task used in the study is a version of active avoidance, the following describes the rationale behind choosing USAA among the various active avoidance tasks.

Avoidance learning (including active avoidance) is generally understood as a two-stage process, with PTC first, then the instrumental phase where animals learn to escape, avoid, or terminate the CS, US, or both by performing actions such as shuttling, wheel turning, and pressing levers (Mowrer & Lamoreaux, 1946; Mowrer, 1947; Miller, 1948, 1951; Brown & Jacobs, 1949; Kalish, 1954; Solomon & Wynne, 1954; Rescorla & Solomon, 1967; McAllister & McAllister, 1971; Overmier & Lawry, 1979; Levis, 1989; Campese et al., 2015).

Because many of these tasks involve both the CS and the US in the instrumental phase, it is difficult to parse out the different kinds of learning taking place simultaneously (Weiskrantz, 1956; Goddard, 1964; Gabriel, Lambert, Foster, Orona, Sparenborg, & Maiorca, 1983; Isaacson, 1982). For example, in a two-way signaled active avoidance (SigAA) task, trials consist of a tone CS that lasts for 15 seconds, where the rats could terminate both the CS and the electric footshock US by shuttling (Choi, Cain, & LeDoux, 2010; Moscarello & LeDoux, 2013; Ramirez, Moscarello, LeDoux, & Sears, 2015). Thus, their shuttling behavior is reinforced by both the termination of the CS and also the elimination of the US. The contribution of these two events are intermixed and hard to separate.

USAA was originally a procedure developed by Sidman (1953) that used dogs as subjects that bypassed the problem of CS exposure in avoidance learning. It has been recently adapted as a task where rats learn to acquire avoidance responses without the presentation of the CS (Lazaro-Munoz, LeDoux, & Cain, 2010; Campese et al., 2015). Details on how USAA is run is exposited in the procedure section under USAA. Using USAA for the instrumental phase sets up the grounds for the PIT task to examine the influence of the Pavlovian CS on the instrumental shuttle responses.

Pavlovian to instrumental transfer (PIT) testing. The ability of the Pavlovian CS to motivate instrumental responses was established in the previous section on active avoidance tasks. Although some tasks like escape from threat (EFT; Cain & LeDoux, 2007; LeDoux, 2014) show promise in studying the motivational aspects in action, PIT has been a more effective mode of studying motivation in action (Estes, 1948; Lovibond, 1983). However, most studies using PIT have been in appetitive studies, and the neural basis of PIT has been examined largely on the appetitive side (Hall, Parkinson, Connor, Dickinson, & Everitt, 2001; Holland & Gallagher, 2003;

Corbit & Balleine, 2005; Balleine & Killcross, 2006; Campese et al., 2013, 2014; McCue, LeDoux, & Cain, 2014). For example, tone CS has been shown to increase lever pressing for food in rats (Holmes, Marchand, & Coutureau, 2013).

In contrast to the abundant studies on appetitive PIT, very little attention has been given to studying aversive motivation using PIT. Rather, investigations into the conditioned suppression task have expanded their interpretations to both appetitive and aversive PIT (Estes & Skinner, 1941; Hunt & Brady, 1951; Killcross, Robbins, & Everitt, 1997). However, conditioned suppression (e.g. a tone CS is presented to the rat, and the rat reduces the rate of lever press for food) is not an accurate comparison for how the CS facilitates avoidance responses (see Campese et al., 2013).

Recently, Campese et al. (2013) have designed an aversive PIT procedure for rats that examines conditioned motivation, and is comparable to appetitive PIT tasks as the US is consistent in both PTC and the instrumental phase (Bolles & Popp, 1964; Rescorla & LoLordo, 1965; Rescorla, 1968; Weisman & Litner, 1969; Overmier & Payne, 1971; Overmier & Brackbill, 1977; Patterson & Overmier, 1981). The specifics of this PIT testing phase are detailed by Campese et al. (2014) as well as in the procedure section under PIT testing. The additional strength of this new aversive PIT paradigm is that it allows for examinations of the neural basis for these behaviors and motivational processes.

Neural Basis of Aversive Motivation

Previous research pinpoints the amygdala as the major site for PTC, and the neural circuitry for the acquisition of the CS-US pairing in auditory PTC within the amygdala is well established (Davis, 1994; Maren & Fanselow, 1996; LeDoux, 2000). The lateral amygdala (LA) is the region where projections from the acoustic thalamus and somatosensory thalamus arrive to

pair auditory CS with the shock US (LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Kapp, Whalen, Supple, & Pascoe, 1992; Amorapanth, LeDoux, & Nader, 2000; Goosens & Maren, 2001; Jimenez & Maren, 2009). Thus, LA serves as the input region for PTC, while the central amygdala (CeA) plays the main output role with intraamygdala connections between the LA and CeA mediating the learning process (Davis, 1992; Pitkanen, Savander, & LeDoux, 1997; Campese et al., 2015).

It has been found in active avoidance (AA) studies (Choi, Cain, & LeDoux, 2010), as well as in EFT studies (Amorapanth et al., 2000), that LA and basal amygdala (BA) but not CeA are key to learning active avoidance. However, contributions of the amygdala disappear with overtraining in AA (Poremba & Gabriel, 1999; Lazaro-Munoz et al., 2010). Campese et al. (2014) found that LA and CeA but not BA are important for PIT. These findings seem to suggest that LA is vital for learning CS-US pairings and reactions, but after that the neural circuits involved in conditioned reactions and conditioned motivation diverge.

This thesis is an exposition of the pilot study that explored the effect of the Pavlovian CS on instrumental response rates using multiple signals for distinct aversive threats, which allowed for the examination of specific vs. general forms of aversive motivation. By selectively suppressing neural activity in CeA and BA, the study looked at the neural circuits involved in these two forms of conditioned motivation. We expect to replicate the Campese et al. (2014) finding that suppressing CeA will reduce PIT, and that inhibiting BA will not have an effect on PIT – for both the shock-paired CS as well as the klaxon-paired CS.

Method

Subjects

All subjects were male Sprague–Dawley rats (Hilltop Lab Animals, Scottsdale, PA) weighing around 300 g at the start of the experiment. Subjects were housed in standard plexiglass solitary cages with paper bedding and were exposed to a 12:12 light:dark schedule. Subjects had free access to food (standard chow) and water while in their home cages. Research was conducted at New York University (New York, NY), and standards of housing and care were approved by the NYU Animal Care and Use Committees.

Apparatus

Subjects were placed in standard conditioning boxes (model H10-11R-TC: Coulbourn Instruments, Whitehall, PA) for PTC, then in two-way shuttleboxes (H10-11R-XX) for USAA and PIT testing. All boxes were equipped with stainless steel grid floors that delivered the scrambled electrical footshock with slidable grey trays underneath it for droppings. In addition, the boxes were equipped with 5 ohm speakers installed on the side walls that presented the 5 kHz tone and noise CSs used in the study, as well as infrared indicator lights that allowed experimenters to see when the cue was on. Both standard conditioning boxes and two-way shuttleboxes were housed in sound-attenuating chambers (models H10-24TA and H10-24A respectively).

The standard conditioning boxes had klaxons (Fun Horn: Manufacturing Corporation, Deer Park, NY) placed on top of the boxes and inside the sound-attenuating chambers. Klaxons are extremely loud horns that can serve as an aversive US. The standard boxes each had a house light and a camera installed in the middle of the ceiling to record movement. The two-way shuttleboxes were divided by a metal panel with a threshold cut away to allow for shuttling. There was a camera in the middle of the ceilings on each section to record movement in the

whole box, as well as a house light on each section's ceilings. Each section also had photocell sensor bars (H20-95X) to detect movement between the two chambers.

Procedure

The procedures for PTC, USAA, and PIT testing have all been established by previous studies (see Campese et al. 2013, 2014). The first part of the experiment consisted of subjects first receiving PTC, then 15 days of USAA training, and ending with four days of PIT testing (Figure 2). This first test phase established the baseline PIT scores and confirmed that both shock and klaxon cues were sufficiently motivating to produce the PIT effect. Surgeries were then performed, with two weeks of recovery time for virus expression (see Surgery section below). The second block of testing began following a single USAA reminder session. Prior to these test sessions, subjects were given intraperitoneal (IP) treatment with Salvinorin-B (Sal-B) or the saline vehicle (see Drugs section). Each subject was given two tests with each cue following each treatment to produce counterbalanced data looking at CS-type and treatment. However, due to the experimenter's oversight, two days of PIT testing were not performed due to accidental replication of previous test assignments. For these data points, only a single session was considered.

Pavlovian threat conditioning (PTC). All subjects were trained with two CS-US arrangements. On day 1, all subjects received CS1 paired with US1, and on day 2, CS2 paired US2. The order and identity of the stimuli and outcomes were counterbalanced. The PTC protocol involved a five-minute baseline, then three CS-US pairings with varying intervals of about three minutes. The total session lasted 15 minutes. The tone and noise CS (5 kHz, 30 seconds) preceded either a footshock (0.5 mA × 1 second) or klaxon (5 seconds) US. The strengths of the footshock and klaxon threats used have been tested prior to this experiment to

ensure they are capable of producing the same degree of conditioned responding (unpublished raw data).

Unsignaled Sidman active avoidance (USAA). After the first two days of PTC sessions, starting day three, subjects underwent 15 days of USAA training over three weeks. Subjects were placed in two-way shuttleboxes, where they received an electric footshock (0.5 mA) every 5 seconds unless they performed a shuttle response (moving from one section of the shuttlebox to the other section through the threshold). This was the designated shock-shock (S-S) interval of 5 seconds, and shuttling during this period was considered an *escape response*. Thus, if the rat shuttled right after the shock, it was considered an escape response. Shuttling delayed the next shock by 30 seconds. If the rat shuttled during this 30-second period (i.e., response-shock or R-S interval), it was considered an *avoidance response*. So the rat would either need to make an escape response first then shuttle again before the end of the R-S interval for an avoidance response or already be in the R-S interval and shuttle again to add another avoidance response. Subjects received a 0.3 second blinking house light feedback with every shuttling response. USAA sessions terminated at the 25-minute mark, and the house lights were turned off.

Rats that failed to demonstrate 20 or more avoidance responses for two consecutive sessions by the tenth USAA session were deemed poor avoiders and were taken out of the study (Lazaro-Munoz et al., 2010). The reminder USAA session conducted in the second part of this study was run in the same manner as the 15 days of USAA training in the first part of the study.

Pavlovian to instrumental transfer (PIT) testing. After day 15 of USAA training, subjects started their four consecutive days of PIT testing. Subjects were placed in the same two-way shuttleboxes used for USAA training, but were not presented with any footshock US throughout the session. Thus, they shuttled under extinction. The response feedback of blinking

house lights to each shuttle response was still presented. When each rat's shuttling rate fell below two responses per minute, one continuous CS (tone or noise, counterbalanced) was presented until ten shuttles were performed. The PIT test ended with the tenth shuttle response (i.e., CS terminated and the house lights turned off). During these tests, response rates were the dependent measure.

The same PIT tests were conducted twice for each CS condition (i.e. each rat would be given the same CS for tests 1 and 2, and the other CS for tests 3 and 4). The scores in these two tests would be averaged for each condition. The stimulus order was counterbalanced in relation to training assignments. These data were then organized in terms of the predicted outcome (i.e., shock or klaxon) and then analyzed (see results below).

In the post-surgical testing phase, a total of eight tests were conducted where drug assignment and stimulus identity were counterbalanced, and these tests were orthogonal to training assignments. This arrangement produced measures of PIT to each CS following Sal-B and vehicle treatment.

Drugs. For the post-surgery PIT tests, subjects were systemically injected with either the drug Sal-B (30mg/kg dissolved in dimethyl sulfoxide or *DMSO*: see Vardy et al, 2015) or saline 30 minutes prior to the PIT tests to allow for the drug to activate the expression of the viruses in target areas. KORD is selectively activated by pharmacologically inert ligand Sal-B. In other words, Sal-B acts as a switch that can turn off the KORD infected neurons, which are otherwise normally functioning. This enabled the inhibition of only the CeA or BA during PIT testing.

Surgery. Subjects were anesthetized with ketamine and xylazine (i.p., 100 mg/kg; 6.0 mg/kg, Phoenix Pharmaceutical). Surgeries were performed on stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Small burr holes were drilled above the target brain areas using

the coordinates based on the rat brain atlas (Paxinos & Watson, 2005). Using 1 µl Hamilton syringes (Hamilton), 1 µl of adeno-associated virus 9 kappa-opioid receptors designer receptors exclusively activated by designer drugs (AAV 9 KOR DREADD, in short, KORD; Vardy et al., 2015) was infused into CeA or BA over five minutes with an additional five minutes before removing the needle.

After 14 days of recovery in home cages, rats received one reminder USAA training session, then were retested.

Verification of viral expressions. Rats were anesthetized with chloral hydrate (150 mg/kg) and were exsanguinated and perfused transcardially with 4% paraformaldehyde (PFA) made in 0.1M phosphate buffer (PB), pH 7.4. The brains were post-fixed in PFA, blocked and cut on a Leica Vibratome into 50 µm sections.

Animals were perfused and tissue sections were collected as described above for immunohistochemistry. Every fifth section of the CeA and BA were processed for the immunohistochemistry (ICC) procedure. Tissues were blocked for 30 minutes in 1% bovine serum albumin (BSA; Sigma-Aldrich) to prevent nonspecific binding of antibodies. Tissues were then incubated overnight in rabbit anti-GFP (1:2000) using 0.2% Triton-X, rinsed 3×, incubated for 30 minutes goat anti-rabbit biotinylated IgG (1:200, Vector Labs, Burlingame, CA), rinsed 3×, and incubated for 30 minutes in avidin–biotin–horseradish peroxidase complex (Vectastain Elite Kit, Vector Laboratories). Following rinses, the reaction product was visualized using the chromogen substrate Very Intense Purple (Vector Laboratories). All incubations were done at room temperature, all rinses used PBS, and incubations in antibodies were made in 1% BSA. Sections were then mounted on gelatinized slides, dehydrated, coverslipped with Permount, and scanned using the Olympus VS120 microscope.

Results

This study began with 24 rats, with five being excluded due to their poor avoidance learning during the USAA phase or missed injection. The averages of the avoidance responses for each of the counterbalanced PTC condition groups over the 15 days of USAA training are depicted in Figure 3. There were no significant interaction among the four groups over the 15 days of USAA training, $F(3,16) = 0.43$, $p > .05$.

Pre-surgery PIT Tests

In the PIT tests (T1-4) before the surgeries, subjects received two sets of two consecutive tone or noise CS PIT tests (e.g. T1&T2 – tone CS, T3&T4 – noise CS). As the impetus behind this study was to examine whether there are differences in the motivational factor of the CS on the instrumental actions (shuttling rate), results for the CS that predicted shock (CS-Shock) and the CS that predicted klaxon (CS-Klaxon) were compared (Figure 4). There was no significant difference in the response rates between the CS-Shock ($M = 1.63$, $SD = 0.55$) and the CS-Klaxon ($M = 1.41$, $SD = 0.44$) conditions during the Pre-CS period, $t(19) = 1.21$, $p > .05$. The avoidance response rates during the CS period were also not significant between the CS-Shock ($M = 5.27$, $SD = 2.65$) and the CS-Klaxon ($M = 5.72$, $SD = 2.65$) conditions, $t(19)= 0.50$, $p > .05$. There was also no significant difference in the change in shuttling rates (CS minus Pre-CS) between the CS-Shock condition ($M = 3.64$, $SD = 2.84$) and the CS-Klaxon group ($M = 4.32$, $SD = 2.83$), $t(19) = 0.68$, $p > .05$.

Post-surgery PIT Tests

The original plan for the post-surgery PIT tests included four sets of two consecutive tone or noise CS PIT tests. However, two sets included only one test each because of data discarded due to treatment errors.

Surgery, by itself, did not significantly change the motivational factor of the CS on the avoidance responses in PIT tests. The difference in the avoidance responses in the CS-Shock condition (CS minus Pre-CS) between the pre-surgery PIT tests ($M = 3.64$, $SD = 2.84$) and post-surgery PIT tests (vehicle trials, BA and CeA group combined, $M = 3.60$, $SD = 3.67$) was not significant, $t(19) = 0.04$, $p > .05$. The CS-Klaxon condition, with pre-surgery PIT tests ($M = 4.32$, $SD = 2.83$) and post-surgery PIT tests ($M = 3.33$, $SD = 4.98$), was also not significant, $t(19) = 1.25$, $p > .05$. Systemic vehicle injections of saline are inert in nature, allowing the comparison with the pre-surgery PIT tests. Thus, the subject's having undergone surgery did not seem to contribute significantly to how the CS motivated their performance in PIT.

Figure 6 compares the avoidance responses in the CS-Shock and CS-Klaxon conditions like the pre-surgery PIT tests (T1-4) but distinguishes the Sal-B test data from the vehicle tests in both the BA target group and the CeA target group. In the BA group, for the CS-Shock condition, there was a significant reduction in PIT from the vehicle condition ($M = 2.88$, $SD = 3.04$) compared to the Sal-B condition ($M = 1.10$, $SD = 0.82$), $t(8) = 1.94$, $p < .05$, one-tailed. One subject was removed from this analysis due to the excessive shuttling when injected with Sal-B. In contrast to the CS-Shock condition, Sal-B did not produce a significant reduction in PIT in the CS-Klaxon (general) condition – vehicle condition ($M = 4.46$, $SD = 6.90$) vs. Sal-B condition ($M = 2.12$, $SD = 2.24$), $t(9) = 1.21$, $p > .05$.

Sal-B inhibition of CeA resulted in significantly reduced PIT in the CS-Shock (specific) condition, where vehicle condition ($M = 4.37$, $SD = 4.36$) and Sal-B condition ($M = 2.52$, $SD = 3.34$), $t(9) = 2.04$, $p < .05$, one-tailed, as well as in the CS-Klaxon (general) condition, where vehicle condition ($M = 2.21$, $SD = 1.39$) and Sal-B condition ($M = 1.40$, $SD = 1.39$), $t(9) = 2.39$, $p < .05$, one-tailed.

Immunohistochemistry

Figure 5 shows the extent of the KORD expression in CeA (left) and BA (right) overlaid across subjects in each group, using rat brain atlas (Paxinos & Watson, 2005) as a guide.

Discussion

This pilot study used a novel behavioral aversive PIT paradigm (Campese et al., 2013) to examine the specific vs. general forms of motivation, as well as the underlying neural circuitry using the newest DREADD technology (Vardy et al., 2015). This study demonstrated again the aversive PIT effect using our new PIT protocol. Furthermore, we were able to confirm our first hypothesis that the CeA is involved in PIT. Both lesioning the CeA (Campese et al., 2014) and suppressing the neural activity in CeA here with KORD impair the aversive CS from motivating the rats to perform USAA. Compared to lesion studies, chemogenetic methods like the one employed in this study using KORD offer a more flexible approach to testing the effects of neural activity in targeted regions of the brain. This allowed us to run several PIT tests with vehicles (control) and Sal-B, and compare the effect of this neural manipulation on PIT within-subjects.

However, inhibiting BA led to unexpected findings. While the suppression of BA did not affect general PIT, it reduced specific PIT. This is contrary to our hypothesis that BA would not be involved in PIT, which was based on the Campese et al. (2014) findings that lesions of BA did not impair PIT. There were several differences between this study and the lesion study. First, the brain manipulations were different (electrolytic lesions vs. viral expressions). However, if viral injections were the cause of the different results, it should have affected the PIT effect in both CS-Shock (specific) and CS-Klaxon (general) conditions. Furthermore, the replication of the CeA results makes it unlikely that effects of the surgery was unique for the CS-Shock

condition for the BA group. Second, the shock intensities were 0.7 mA for the previous study, while this project used 0.5 mA. The shock intensity for this experiment was chosen so that CS-Shock and CS-Klaxon would produce comparable PIT effects (see Figure 4).

The only key difference between the lesion study and this study is then the introduction of another CS-US relationship. Moreover, the surprising aspect of our finding is that specific PIT, which uses CS-Shock and is thus similar to the lesion study where there was only tone-shock pairing, had different results instead of general PIT. It is unclear whether the recruitment of BA was due to subjects in the CS-Shock condition (specific) having to process more information.

Because subjects are exposed to only shock during the USAA phase and CS-Shock is associated with oncoming shock, it seems unlikely at first that the CS-Shock condition requires more processing than the CS-Klaxon condition during PIT tests. Given that the subject's avoidance response has never been reinforced by the removal of the klaxon US, the subject's increased avoidance response when it is presented the CS-Klaxon would likely involve another step, in the form of stimulus generalization (where shuttling becomes a response to all aversive auditory stimulus; Delamater, 1998; Delamater, Kranjec, & Fein, 2010). However, stimulus generalization may be acquired during the USAA phase when subjects learn to respond to threats by performing avoidance responses through acquired equivalence processes (Honey & Hall, 1989; Delamater, 1998; Ward-Robinson & Hall, 1999; Coutureau, Killcross, Good, Marshall, Ward-Robinson, & Honey, 2002). The presentation of CS-Klaxon in the PIT tests then provides the opportunity and motivation for subjects to demonstrate their previous learning of stimulus generalization. Furthermore, a generalized response to any threat provides a quicker response and therefore requires less processing than a sensory-specific case, which is in line with the current results.

The appetitive studies provide a good comparison for this pilot study, as the role of basolateral amygdala (BLA) and CeA in PIT has been studied extensively in the appetitive domain (Blundell, Hall, & Killcross, 2001; Hall, Parkinson, Connor, Dickinson, & Everitt, 2001; Balleine, Killcross, & Dickinson, 2003; Holland & Gallagher, 2003). While we focused on BA, other investigations often do not make the distinction between BA and LA, and group them together as BLA. Specific vs. general PIT in the appetitive domain showed a double dissociation between BLA and CeA (Corbit & Balleine, 2005). Like the results in this study, BLA lesions abolished outcome specific motivational effects of the CS, while general motivational effects were not affected. While Balleine et al. (2003) and Blundell et al. (2001) reported reduction in specific PIT when BLA was lesioned, Hall et al. (2001) and Holland and Gallagher (2003) claimed lesioning BLA had no effect on PIT. Corbit and Balleine (2005) pointed out that this discrepancy could arise from having two actions rewarded by different outcomes as opposed to having one action and seeing the influence of a single excitatory cue on performance. These findings seem to mirror the Campese et al. (2014) and this study's findings on BA, where lesioning BA has no effect when there is one CS-US pairing, but affects specific PIT when there are two CS with two different threat outcome-representations.

However, the appetitive and aversive PIT studies clash when it comes to the role of CeA. The results of the current study suggest that for aversive general motivation, CeA is needed while BLA is not, and both CeA and BLA are employed for sensory-specific PIT. In contrast, Corbit and Balleine (2005) found that while CeA is needed for appetitive general PIT, BLA is not, and CeA is not needed for specific PIT while BLA is needed. In other words, there's a dissociation in the appetitive domain where general motivation is mediated by CeA and specific motivation is mediated by BLA, which has led some to argue for a parallel processing of

motivation in the amygdala (Balleine & Killcross, 2006). In the aversive domain, however, evidence points to a more nuanced picture where specific motivation is serially processed (both CeA and BA are needed, thus the LA-BA-CeA pathway) and general motivation is processed by the LA-CeA pathway. Therefore, not only do the neural substrates of motivation seem to differ between appetitive and aversive domains, but sensory-specific and sensory-general processing of aversive motivation seem to differ as well.

Depending on the tasks, aversively motivated instrumental reactions and actions utilize the LA-BA pathway (AA, EFT, USAA) or LA-CeA (general PIT) and LA-BA-CeA pathways (specific PIT). The characterization of LA-BA as the informational route, and LA-CeA as the emotional/affective route may help explain this variety in neural circuitry (Campese et al., 2015). Tasks involving the learning of active avoidance behaviors would recruit BA, while CeA might be involved in reactions. Perhaps, generalized responses to the aversive CS is reactive in nature, which would explain the sensory-general PIT results, while sensory-specific CS solicits both the informational BA area as well as the reactive response CeA area.

In conclusion, this investigation used a novel aversive PIT paradigm and combined it with new DREADD technology that allows temporary suppression of neural activity in selected regions to find evidence that general and specific forms of CS-driven motivation of avoidance responses utilize different neural pathways within the amygdala. Further studies that parse out the informational pathway from the emotional/affective pathway would help bridge the connections between threats and our responses.

The results of this study seem to affirm the idea that aversively motivated responses to threat are processed in different ways depending on stimuli. The role of the amygdala circuitry in

aversive motivation of responses to threat provide insight into clinical issues regarding overcoming fears, phobias, and anxiety.

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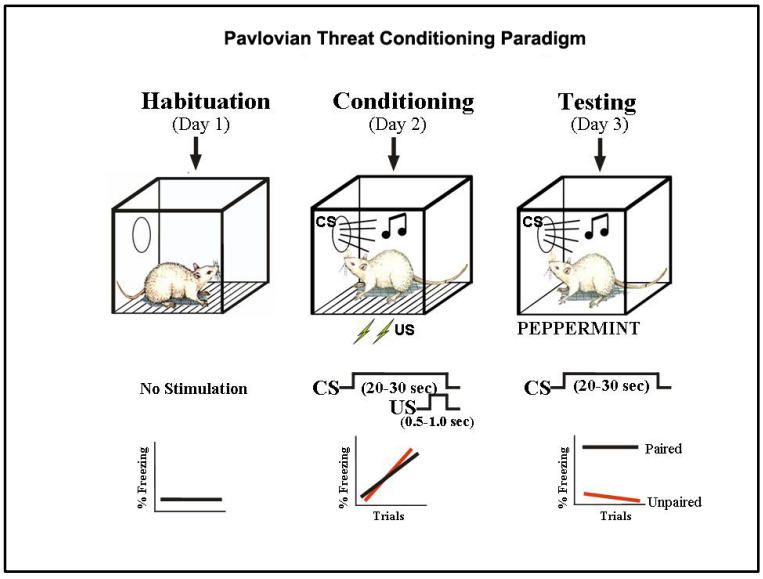

Figure 1. Pavlovian threat conditioning (PTC). The habituation phase involves placing the rat in the conditioning box without any CS or US. In the conditioning phase, the rat is exposed to 20 to 30 seconds of auditory CS (tone or noise), before the US (electrical scrambled footshock) is administered. This CS-US pairing is repeated several times. The rats learn to associate the CS with the US and start freezing to the CS. On the test day, the rat is placed in a conditioning box with different contextual cues (using different floors and scent). When the rat is presented with the CS, experimenters can score the duration of freezing. If the rat has not been administered the CS-US pairings (unpaired control condition), there is no freezing response to the CS. This Reprinted from The LeDoux Lab, J. LeDoux, 2016. Retrieved from http://www.cns.nyu.edu/labs/ledouxlab/research.htm. Copyright 2015 by LeDoux Lab CNS.NYU. Reprinted with permission.

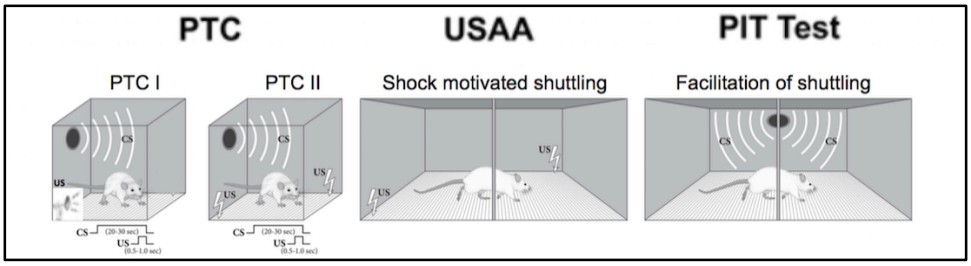

Figure 2. In PTC sessions, the rat was placed in a standard conditioning box with an electrified grid (for footshock) and a speaker for the CS (tone or noise). Each rat had two CS-US pairings (e.g. Day 1: tone-footshock, Day 2: noise-klaxon; counterbalanced). In the USAA training, rats were placed in a two-way shuttlebox, also with an electrified grid. Rats prolonged the footshock by shuttling between two chambers. For the PIT test, rats were again placed in a two-way shuttlebox but there was no footshock and one CS presentation until the termination of the test. Reprinted from The LeDoux Lab, J. LeDoux, 2016. Retrieved from http://www.cns.nyu.edu/labs/ledouxlab/research.htm. Copyright 2015 by LeDoux Lab CNS.NYU. Reprinted with permission.

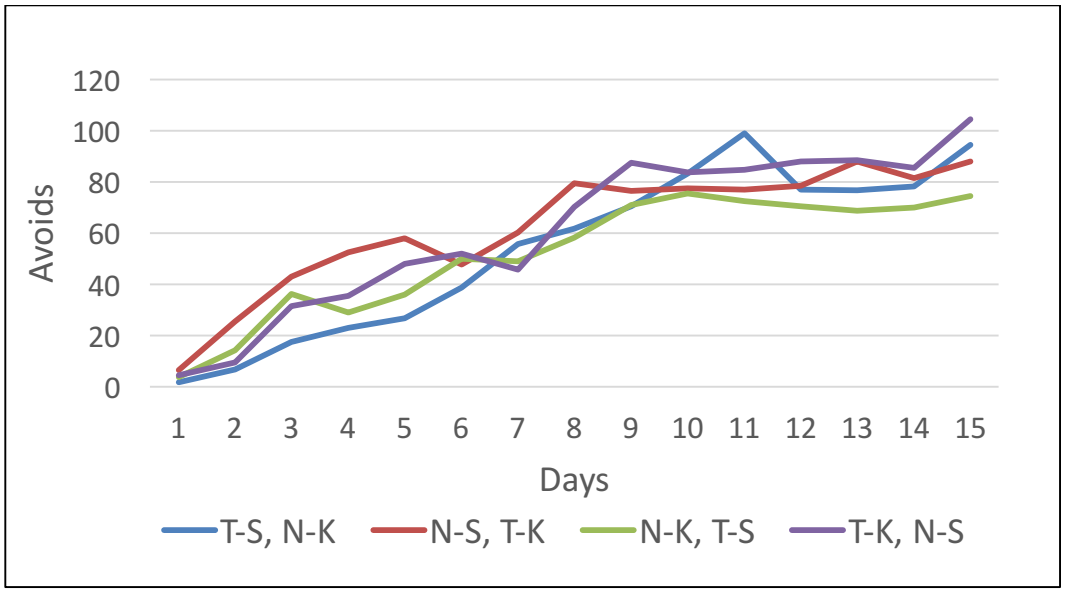

Figure 3. Avoidance responses in the USAA phase. Each line represents one of the four PTC conditions. Avoidance responses of the rats in each group are averaged for each of the 15 USAA sessions. T (tone), S (shock), N (noise), K (klaxon).

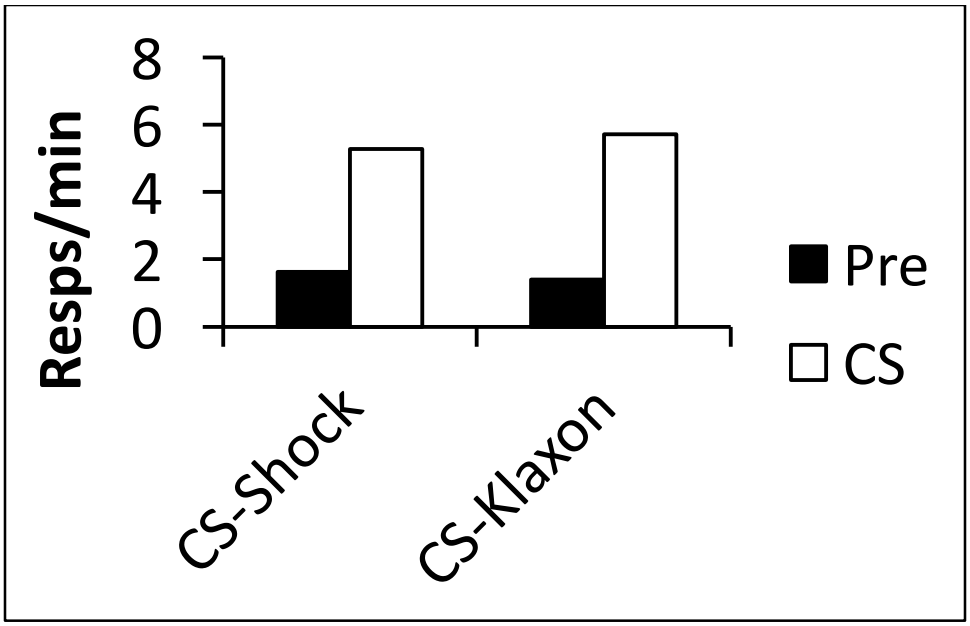

Figure 4. Pre-surgery PIT tests. Comparisons between the response rates (shuttle responses per minute) when the CS (tone or noise) that was linked with shock or klaxon was presented during PIT tests 1~4. The black Pre-CS bars refer to the response rates right before the CS was presented where its duration was set by how long the CS was presented during the PIT test. The white CS bars refer to the response rates while the CS was on.

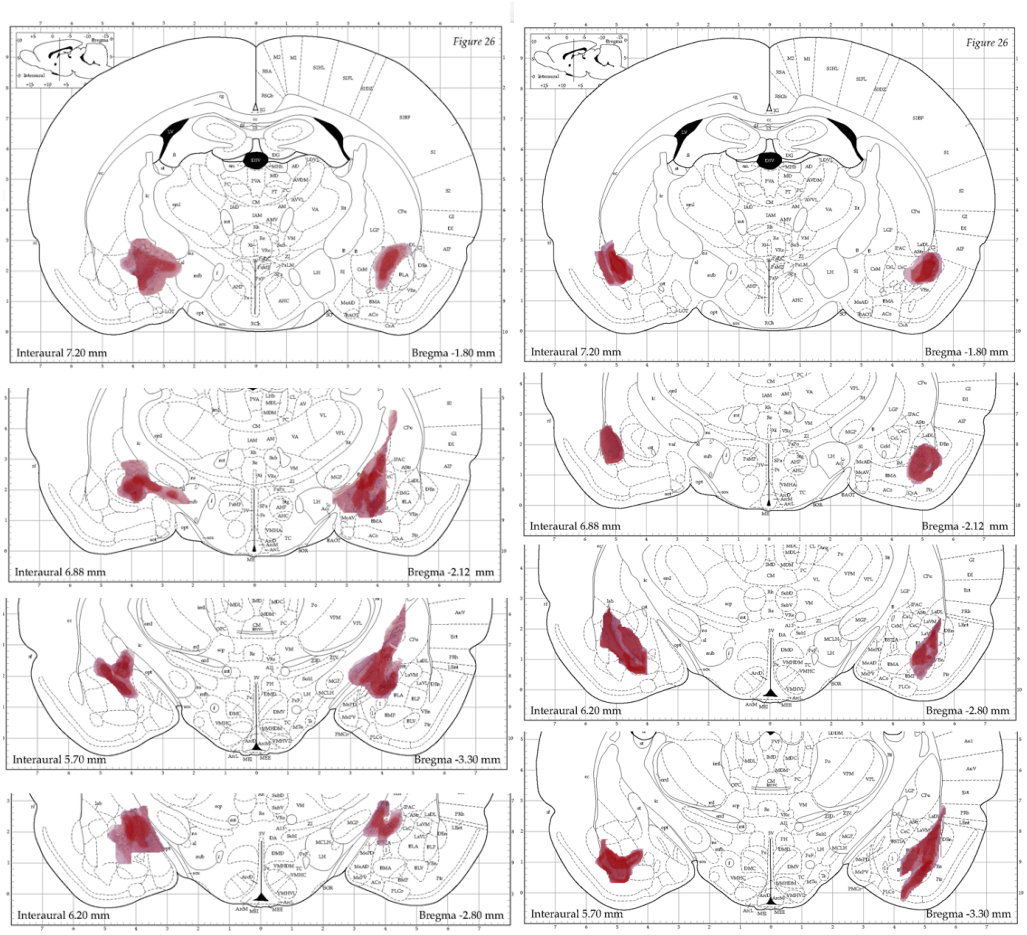

Figure 5. Extent of KORD expression in CeA (left) and BA (right) overlaid across subjects in each group. Used rat brain atlas (Paxinos & Watson, 2005) as guide.

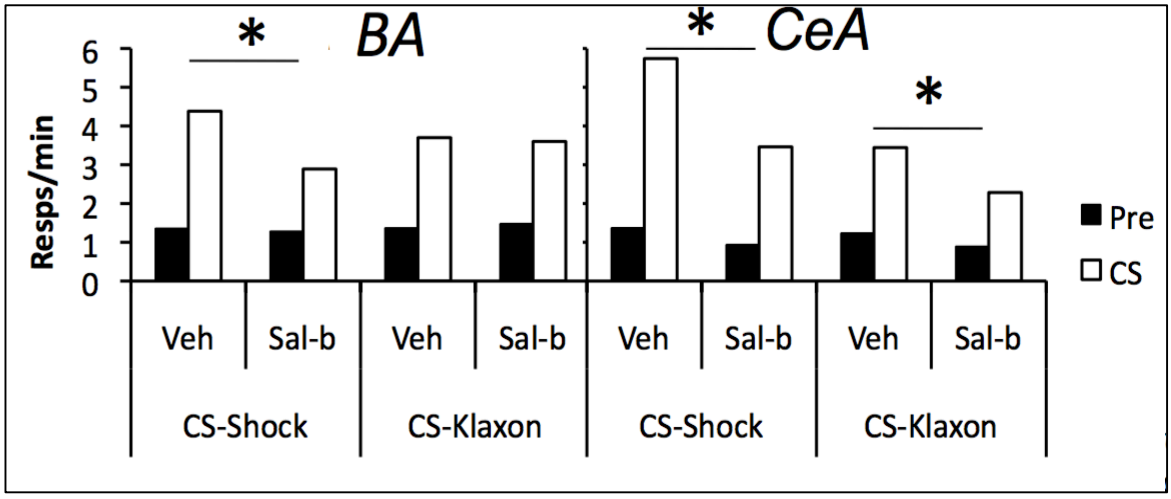

Figure 6. Post-surgery PIT tests. Comparisons between the response rates (shuttle responses per minute) when the CS (tone or noise) that was linked with shock or klaxon was presented during PIT tests 5~10 for BA and CeA groups. Vehicle condition serves as control for the Sal-B condition where Sal-B inactivates KORD infected cells that inhibit the neural activity in the target areas (BA or CeA). The black Pre-CS bars refer to the response rates right before the CS was presented where its duration was set by how long the CS was presented during the PIT test. The white CS bars refer to the response rates while the CS was on.