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Dopamine d1 receptor activity in the basolateral amygdala is important for mediating fear, reward and safety discrimination learning

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DOPAMINE D1 RECEPTOR ACTIVITY IN THE BASOLATERAL AMYGDALA IS IMPORTANT FOR MEDIATING FEAR, REWARD AND SAFETY DISCRIMINATION LEARNING

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ABSTRACT

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Post traumatic stress disorder (PTSD) patients frequently show impairment in safety learning (Jovanovic, Kazama, Bachevalier, & Davis, 2012). Since the amygdala is known to be critical for emotional processing(Wassum & Izquierdo, 2015) and dopamine signaling in the amygdala is important for mediating both fear and reward learning, current experiments examined the role of dopamine signaling in the BLA in mediating both safety learning and reward seeking. We manipulated dopamine D1 receptor activity with a D1 receptor agonist (SKF 38393) or D1 receptor antagonist (SCH23390) either systemically or infused directly into the BLA 20 minutes prior to training rats in a fear-safety-reward cue discrimination learning task (Sangha, Chadick, & Janak, 2013). Systemic administration of either the D1 receptor agonist or antagonist impaired fear and safety discrimination learning. The systemic administration of the D1 receptor agonist, but not the antagonist, also impaired discriminative fear and discriminative reward seeking. BLA infusion of the agonist, but not the antagonist, replicated the impairment in fear and safety discrimination learning but not discriminative reward seeking. This study demonstrates that D1 receptor activity in the

BLA is not needed for fear learning, safety learning or discriminative reward seeking, but an increase in dopamine D1 receptor activity within the BLA impairs fear suppression in the presence of the safety signal.

INTRODUCTION

Current drug therapy for posttraumatic stress disorder (PTSD) lacks specificity, and more targeted treatment is needed. The results of a 2016 meta-analysis study indicate that SSRI and SNRI antidepressants are most commonly recommended for the treatment of PTSD (Lee et al., 2016). The two most effective medications, Venlafaxine and Nefazodone, lack sustained benefits and can trigger severe side effects, respectively (Lee et al., 2016). A better understanding of the underlying neural circuit would allow the development of more specific drugs to rescue dysregulations in fear suppression. This approach may lead to the development of drugs with longer lasting benefits upon treatment completion and with less side effects. Given that PTSD affects approximately 8.7% of the general population within their lifetime(Kessler et al., 2005), there is a critical need to map out the neural circuitry underlying emotional regulation to stimulate development of more targeted drug treatments. PTSD patients have an inability to discriminate between danger and safety (Jovanovic et al., 2009), and they have impairments in fear extinction(Morriss, Christakou, & Van Reekum, 2015). Current literature has mostly focused on the reward and fear learning circuits separately. Integrating the safety circuit with the fear and reward circuit is essential for understanding how safety signals elicit approach behaviors and inhibit avoidance behaviors. Without an understanding of the integrated fear-reward-safety circuitry, we

will continue to have knowledge gaps with respect to fear dysregulation by safety signals in PTSD patients.

Overview of Neural Circuitry of Emotion Learning (Figure 1)

The amygdala is critical for both fear and reward learning (Wassum & Izquierdo, 2015). It receives input from the sensory insular cortex (SI)(Kong, Monje, Hirsch, & Pollak, 2014), prefrontal cortex (PFC) (Vertes, 2004), hippocampus (Ishikawa & Nakamura, 2006), and ventral tegmental area (VTA)(Abraham, Neve, & Lattal, 2014). Inputs from those brain structures into the amygdala are critical in mediating complex emotional learning. The SI receives sensory input from different modalities and is important for safety expression (J. P. Christianson et al., 2008). The infralimbic cortex (IL) of the PFC is important for fear extinction(Kong et al., 2014). The hippocampus is important for fear extinction (Corcoran & Maren, 2001). The dorsal hippocampus, in addition to being involved with fear learning (Rossato, Bevilaqua, Izquierdo, Medina, & Cammarota, 2009), is also involved with reward learning (Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011). Similar to the amygdala, the VTA also responds to both valences. Dopamine neurons in the dorsal portion of the VTA are responsive to rewards and dopamine neurons in the ventral portion are responsive to aversive events (Abraham et al., 2014). All of these inputs converge in the amygdala and will affect the outputs to the nucleus accumbens (NAc) (Wassum & Izquierdo, 2015) and periaqueductal gray (PAG)(Herry & Johansen, 2014), among others, for behavioral expression. The NAc mediates both appetitive and avoidance behaviors (Richard & Berridge, 2011) and the PAG is necessary for conditioned freezing expression(Pape & Pare, 2010).

General Overview of the Amygdala in Emotion Learning

The amygdala can be roughly divided into central amygdala (CeA) and basolateral amygdala (BLA). This structure is important for detecting salient stimuli, and eliciting approach or avoidance behaviors in response to either reinforcing or aversive environmental signals (Janak & Tye, 2015; Weymar & Schwabe, 2016). The CeA, which is divided into the lateral portion (CeL) and medial portion (CeM), is made up of mostly GABAergic neurons(Janak & Tye, 2015). The CeA is necessary for fear expression and conditioned orientating responses to the reward CS. It receives direct input from the BLA as well as indirect BLA input via GABAergic intercalated cells located between the BLA and CeA (Amano, Unal, & Paré, 2010; Janak & Tye, 2015). In contrast, the BLA which includes the basal amygdala (BA), basomedial amygdala (BM) and lateral amygdala (LA) is mostly made up of glutamatergic principal neurons and inhibitory interneurons (Janak & Tye, 2015). Glutamatergic principle neurons make up approximately 80 percent of the cells in the BLA, and they make connection with each other through excitatory projections. They also relay inputs from the LA to the BM. The remaining 20 percent are GABAergic interneurons (Pape & Pare, 2010). The BLA is critical for fear expression (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011) and avoidance expression (Bravo-Rivera, Roman-Ortiz, Brignoni-Perez, Sotres-Bayon, & Quirk, 2014). There is evidence showing that fear memories are stored within the BLA. Reactivation of fear memories followed by infusion of a protein synthesis inhibitor, anisomycin, into the BLA disrupts memory in future tests. This effect is observable for both newly formed and older memories (Nader, Schafe, & Le Doux, 2000).

The LA is an important sensory input area of the BLA for receiving both conditioned stimulus (CS) and unconditioned stimulus (US) information. CS input enters the LA through projections from the medial geniculate nucleus(MGn) and auditory cortex (AC) (Tsvetkov, Carlezon, Benes, Kandel, & Bolshakov, 2002). Optical stimulation of inputs from AC and MGn to the LA can effectively replace an auditory CS during fear conditioning (Nabavi et al., 2014). There are at least two sources of plasticity within this pathway during learning. It has been demonstrated that the synaptic input from the AC to the LA shows long term potentiation(LTP) during fear conditioning (Tsvetkov et al., 2002). Inducing long term depression (LTD) or inducing LTP at the synaptic input to the LA following paired training can abolish or reactivate conditioned suppression, respectively (Nabavi et al., 2014). There is evidence showing that the learning induced LTP in the synapse from auditory cortex to the LA is attributed to the increase in presynaptic neurotransmitter release probability, but not quantal amplitude (Tsvetkov et al., 2002). Stress exposure can enhance this LTP by increasing the number of postsynaptic NMDA receptors in LA principal neurons leading to the overall facilitation in fear consolidation (Suvrathan et al., 2014). This fear facilitation might contribute to the resistance in fear extinction observed in individuals with anxiety disorders.

In addition to synaptic changes in the inputs to the LA, plasticity can also be observed downstream within the LA. During fear conditioning, the LA shows an increase in short latency activity to the auditory CS. This effect can be observed in as little as five fear conditioning trials and the increase in LA activity persists even when conditioning continues for 70 more trials. This further supports the idea that fear

memories are established and stored within the LA (Maren, 2000). In addition, the LA receives US input from the spinal/ trigeminal dorsal horn through the periaqueductal gray(PAG) during fear conditioning (Herry & Johansen, 2014). The optical stimulation of LA pyramidal neurons within the amygdala can effectively replace a footshock US and cause animals to subsequently show conditioned freezing to an auditory CS that was previously paired with the optical stimulation (Johansen et al., 2010). Taken together, this pairing of CS and US leads to the strengthening of CS input to the LA during fear conditioning, and it becomes easier for the CS to activate the LA without the unconditioned stimulus (US) input to the LA (Janak & Tye, 2015). The finding that LA activation can serve as an effective US, producing conditioned freezing responses, supports the idea that CS activation of LA pyramidal neurons during conditioning is critical for driving fear memory formation (Johansen et al., 2010).

In addition to fear learning, the amygdala plays a role in reward learning. It encodes reward value and neurons in the amygdala respond to the visual presentation of food only when it's palatable (Wassum & Izquierdo, 2015). The neurons in the BLA have been found to encode information for reward anticipation and reward contingency (Hernádi, Grabenhorst, & Schultz, 2015; Sugase-Miyamoto & Richmond, 2005). BLA neurons also show increases in firing to the reward cue during paired training (Tye & Janak, 2007), and encode changes in reward value by encoding both positive and negative prediction error(Wassum & Izquierdo, 2015). With respect to reward, there is a population of BLA neurons that are responsive to the cue only when the animal is seeking reward, and a different population of BLA neurons that are responsive to the reward cue regardless if the animal is seeking reward (Tye & Janak,

2007). Projections from the BLA to the NAc shell (NAs) is necessary for reward seeking extinction expression(Millan & McNally, 2011) .

Taken together, the BLA regulates both positive and negative emotions. This idea was also supported by a recent publication from our lab looking at fear-reward-safety discrimination learning in rats. We found that the BLA contains fear neurons that show selective responses to the fear cue, safety neurons that show selective responses to the safety cue, and safety + reward neurons that show selective responses to the safety and reward cues (Sangha, Chadick, & Janak, 2013). In addition, the BLA receives projections from the prelimbic cortex (PL) and the infralimbic cortex (IL)(Vertes, 2004). Follow up local inactivation studies from our lab have shown that the PL is necessary for fear expression and discriminative reward seeking, and the IL is necessary for fear and safety discrimination (Sangha, Robinson, Greba, Davies, & Howland, 2014). These findings suggest that inputs from upstream BLA structures may contribute to the learning related changes observed in the BLA during fear, safety and reward discrimination.

Dopamine Signaling

Midbrain dopamine neurons are predominantly located in the VTA, substantia nigra pars compacta (SNc) and retrorubral field (RRF) (Lammel, Lim, & Malenka, 2014). They are responsive to reward cues and to the unexpected delivery of rewards (Schultz, 2013). Past studies have identified dopamine neurons based on the presence of I_h current and responsiveness to dopamine application. However, not all dopamine neurons show these characteristics(Lammel et al., 2014). In addition, dopamine

neurons can be GABAergic or glutamatergic and therefore can release GABA or glutamate in addition to dopamine(Lammel et al., 2014).

A typical dopamine response can be divided into two components. The faster stimulus detection component is important in directing attention and allowing a quicker response to the potential reward. The magnitude of this component is dependent on the intensity of the signal, similarity with other reward signals, presentation context, and novelty of the cue (Schultz, 2016). The slower component encodes prediction error, which reflects the difference between expected and obtained. An increase in firing can be observed when an obtained reward is higher than expected, and decreased firing can be observed when an obtained reward is lower than expected. No change is observed when an obtained reward is fully expected (Schultz, 2013, 2016). This prediction error serves as a teaching signal and provides feedback about changes in reward delivery to drive learning. Mimicking a prediction error by activating dopaminergic neurons during reward delivery leads to an enhancement in cue driven reward seeking (Steinberg et al., 2013). In addition, midbrain dopaminergic neurons are responsive to unpleasant stimuli. Since this increase in firing occurs at the end of an aversive stimulus, this might be due to the reinforcing effect from removal of the aversive stimulus (Schultz, 2013).

Dopamine Receptors

There are five subtypes of dopamine receptors: D1, D2, D3, D4 and D5. Binding of dopamine to D1 or D5 receptors activates stimulatory G proteins and leads to the stimulation of adenylate cyclase activity and production of cyclic adenosine monophosphate (cAMP). In contrast, binding of dopamine to D2, D3 or D4 receptors

activates inhibitory G proteins and decreases adenylate cyclase activity (Abraham et al., 2014). Dopamine receptors can be found throughout the brain. Dopamine D1 and D2 receptors are both expressed in the PFC, NAc, intercalated cells of the amygdala, hippocampus, and striatum. D2 receptors are more selectively expressed in the CEA, substantia nigra, and VTA, whereas D1 receptors are more selectively expressed in the BLA (Weiner et al., 1991). Dopaminergic signaling within the BLA is important for both fear and reward learning. Within the BLA, dopamine levels increase during fear conditioning (de Oliveira et al., 2011) and D1 receptor activity within the BLA is needed for the acquisition of fear extinction(Hikind & Maroun, 2008). Both D1 and D2 receptor activity modulates risk decisions during a reward uncertainty task(Larkin, Jenni, & Floresco, 2016).

Dopaminergic Circuits

The VTA is a critical upstream structure since it sends dopaminergic projections to diverse brain regions regulating both reward and aversive learning. The VTA has projections to both the prelimbic (PL) and infralimbic (IL) cortex through the mesocortical pathway (Abraham et al., 2014). The PL and the IL in turn project to the BLA (Vertes, 2004). Local inactivation studies have shown that the PL is necessary for fear expression and discriminative reward seeking, and the IL is necessary for fear and safety discrimination(Sangha, Robinson, et al., 2014). The VTA also has direct projections to the BLA, CEA, hippocampus and the NAc through the mesolimbic pathway (Abraham et al., 2014). This projection from the VTA to the BLA is important for fear learning. Normally, dopamine levels in the BLA increase during fear conditioning (de Oliveira et al., 2011). An increase in D2 receptor activity in the VTA

has been shown to block both conditioned freezing and the learning related increases in dopamine levels within the BLA (de Oliveira et al., 2011). The BLA also receives a direct projection from the ventral hippocampus (Ishikawa & Nakamura, 2006), as well as an indirect projection from the ventral hippocampus (Verwer, Meijer, Van Uum, & Witter, 1997) via the PFC (Brinley-Reed, Mascagni, & McDonald, 1995). Dopamine receptor activity in the NAc has also been implicated in regulating opposing emotions. D1 receptor activity is needed for consumption behavior in the anterior portion of the NAc. Both D1 and D2 receptor activities are needed for the expression of fear behaviors in the posterior NAc (Richard & Berridge, 2011). In addition, the VTA also projects to the dorsal striatum through the nigrostriatal pathway (Abraham et al., 2014). Dopaminergic neurons within the dorsal striatum are needed for discriminative reward learning (Eagle, Olumolade, & Otani, 2015). It has also been demonstrated that cocaine administration leads to plasticity in the VTA to medial NAc pathway, and painful injection, such as formalin leads to plasticity in the VTA to mPFC pathway (Lammel et al., 2014). Taken together, these data suggests that dopamine signaling is implicated in both fear and reward learning.

Dopamine in Learning

Dopamine is critical for both LTP and LTD in certain brain structures. In the striatum, D1 is necessary for LTP and D2 is necessary for LTD (Schultz, 2013). The VTA also provides necessary dopamine signals to other downstream structures such as PFC, amgydala and NAc contributing to different aspects of fear learning. D1 receptor activity in the amygdala is critical for fear learning (Abraham et al., 2014). D1 receptor activity in the dorsal hippocampus and BLA are necessary for the acquisition of

contextual fear conditioning(Heath et al., 2015). In addition, restoring dopamine function in the NAc and BLA in mice with impaired dopamine synthesis can rescue impairments in long term memory of fear potentiated startle. This demonstrated that dopamine signaling in the NAc and BLA is necessary for forming long term memory of fear potentiated startle (Fadok, Darvas, Dickerson, & Palmiter, 2010). Dopamine is also involved with fear extinction. Dopamine levels in the PFC typically increase during fear extinction training. Blocking D1 receptor activity after or blocking D2 receptor activity before extinction training in the IL impairs fear extinction consolidation (Abraham et al., 2014).

Dopamine D1 signaling in the amygdala, Nac, PFC and hippocampus, are implicated in reward learning. D1 receptor activity in the NAs, dmPFC and BLA is necessary for the reinstatement of drug seeking during stress(Tobin, Sedki, Abbas, & Shalev, 2013). D1 receptor activity in the BLA, CeA, and ventral subiculum of the hippocampus is necessary for the acquisition of conditioned approach behavior (Matthew E. Andrzejewski & Ryals, 2016). D1 receptor activity in the BLA and CeA is needed for the acquisition of conditioned lever press (M. E. Andrzejewski, Spencer, & Kelley, 2005).

Dopamine release in the NAc is also important in reward learning. Inappropriate dopamine release in the NAc is linked to drug abuse (Lammel et al., 2014). NAc dopamine release is modulated by learning. It has been shown that learning results in a larger dopamine release, but overtraining decreases the learning related facilitation in dopamine release(A. L. Collins et al., 2016). During discriminative reward learning, the NAc also shows a pattern of enlarged dopamine

release to the discriminative stimulus (Jones et al., 2010). This learning related increase in dopamine level in the NAc is amygdala dependent, and it can directly affect reward-seeking behavior. Ipsilateral BLA inactivation prior to training can diminish the learning related increase in dopamine level to the discriminative stimulus and also impaired approach behavior to the port (Jones et al., 2010).

There is a glutamatergic projection from the BLA to NAc. Optical activation of this pathway is reinforcing because rats will learn to enter the port for the optical stimulation of this pathway. Optical inhibition of the same pathway decreased cue driven reward consumption(Stuber et al., 2011). D1 receptor activity in the NAc is needed for the stimulation to be effective because animals with D1 antagonist infusion into the NAc showed a decrease in cue-driven nose poke activity to the active port (Stuber et al., 2011). This demonstrated that the optical induction of reward seeking behavior is mediated by dopamine D1 receptor activity in the BLA-NAc projection. Taken together, dopamine signaling from the VTA affects a wide range of brain structures in regulating both reward and aversive learning.

Fear Conditioning

In Pavlovian fear conditioning, the conditioned stimulus (CS) signals the occurrence of a threat(LeDoux, 2014). The CS is usually a tone or light and the threat US is usually a mild footshock. The repeated pairing of the two results in a learned defensive response to the CS in anticipation to the threat (LeDoux, 2014). For an auditory CS, sensory information travels from the inferior colliculus (IC) through the posterior intralaminar (PIN) and medial division of medial geniculate nucleus (MGm) into the LA (Pape & Pare, 2010). It can also travel indirectly from MGm or ventral

division of medial geniculate nucleus (MGv) to LA through the auditory cortex (Herry & Johansen, 2014). US information travels from the spinal/trigeminal dorsal horn through the PAG and converges with CS information in the LA (Herry & Johansen, 2014). Fear learning induces plasticity within the LA prior to the observed CS facilitation in auditory cortex and auditory thalamus. The facilitation in LA is associative because paired training leads to higher CS responding than unpaired presentations. Even in discrimination training, CS facilitation in LA is only observed toward CS and not to the CS- that signals the absence of the US (D. R. Collins & Paré, n.d.). The LA has indirect projections to the CEm via the CEl, BA and ITC cells(Pape & Pare, 2010). During fear consolidation and reconsolidation, theta activity has been shown to synchronize between the dorsal hippocampus (CA1) and BLA. This CA1-BLA activity is then phase locked with the IL during fear extinction (Lesting et al, 2011; Sangha et al 2009; Narayanan et al 2008). Lastly, CEA output is necessary for producing different conditioned fear behaviors. Outputs to the lateral hypothalamus and PAG selectively affects blood pressure and conditioned freezing, respectively(LeDoux, Iwata, Cicchetti, & Reis, 1988).

Reward Conditioning

There is no standard behavioral paradigm for studying reward learning. However, reward learning typically contains a CS such as a tone or light that signals the occurrences of a reward delivery, or signals an opportunity that executing a certain behavior results in the delivery of reward. Reward learning is an amygdala dependent activity. While the CEA is required for reward learning that involves forming associations between the CS and a broader anticipatory response such as approach

behavior, arousal, and changes in heart rate (Balleine & Killcross, 2006), the BLA is required for reward learning that involves forming associations between the CS and the specific aspects of US consumption responses, such as licking and chewing (Balleine & Killcross, 2006). The BLA is also needed when a specific reward outcome is required to guide the operant response (Wassum & Izquierdo, 2015). Although lacking evidence, it has been suggested that the general arousal and anticipatory responses mediated by the CEA may facilitate US specific reward learning that is BLA-mediated (Balleine & Killcross, 2006). In addition, the BLA has projections to other brain structures that can influence different aspects of reward learning. Projections to the dorsal medial striatum (DMS) and insular cortex affect learning of operant response-outcome association. Projections to the OFC affect impulsivity, and disruption in this pathway shifts the preference to choosing a smaller and quicker reward over a larger delayed reward(Wassum & Izquierdo, 2015).

Conditioned Inhibition

A conditioned inhibitor is an inhibitory CS that signals the absence of the US. It is distinguished from the lack of excitation in that it is an active suppression process (Schwartz, Wasserman, & Robbins, 2002) that must pass both a summation test and retardation test(J. P. Christianson et al., 2012). In contrast, a conditioned exciter signals the occurrence of the US. This allow the animal to anticipate in advance. A summation test can be performed by presenting a compound stimulus consisting of both the conditioned exciter and conditioned inhibitor. The compound stimulus should elicit a smaller magnitude of conditioned responding than conditioned exciter alone. The retardation test uses the conditioned inhibitor as a conditioned exciter to be paired

with the US. Pairing the US with the previously used inhibitor should result in slower learning (J. P. Christianson et al., 2012).

Safety Conditioning

Safety cues signal the absence of a threat. This can be induced with unpaired training, backward conditioning, discrimination training or avoidance training (Kong et al., 2014). A learned safety cue is a type of conditioned inhibitor and is an active fear suppressor that can be verified with the summation and retardation tests (J. P. Christianson et al., 2012). In addition, since the fear and reward systems reciprocally suppress each other, suppression of fear via a safety cue will indirectly excite the reward system (Dickinson & Michael, 1979; Dickinson & Pearce, 1977; Gray, 1987), providing learned safety cues with reinforcing properties (J. P. Christianson et al., 2012). It has been demonstrated that animals would preferentially spend more time in the chamber that has a safety signal playing (Rogan, Leon, Perez, & Kandel, 2005). In addition, animals will preferentially choose a lever that also delivers the safety signal when provided with two levers that can both prevent shock occurrence (A. B. P. Fernando, Urcelay, Mar, Dickinson, & Robbins, 2014). Presenting a safety signal has been shown to encourage exploratory behavior to the aversive center region in an open field task (Rogan et al., 2005) and safety conditioning has been shown to rescue impairments in social exploration resulting from prior exposure to inescapable tail shocks(J. P. Christianson et al., 2008).

The amygdala and striatum have opposing functions in safety and fear learning. Imaging studies have identified that the amygdala shows more activity to the fear CS and a decrease in blood oxygenation level dependent activity to the safety CS. The

striatum shows the opposite, with more activity to the safety CS and increased blood oxygenation level dependent activity to the safety CS (Kong et al., 2014). This is consistent with electrophysiological data demonstrating that the LA shows decreases in CS evoked field potential amplitude and slope during safety training, and an increase in CS evoked field potential amplitude and slope during fear training (Rogan et al., 2005). This study also demonstrated that the caudoputamen(CP) shows an increase in CS evoked field potential amplitude and slope during safety training, but no change to fear conditioning. The SI is another area critical for safety expression. It has projections to the amygdala (John P Christianson & Greenwood, 2014). . The hippocampus may be another structure critical for safety. There is evidence that neurogenesis within the hippocampus is necessary for safety conditioning (Pollak et al., 2008). In addition, lesion studies have demonstrated that NAc is not necessary for safety learning and expression(Josselyn, Falls, Gewirtz, Pistell, & Davis, 2005). However, this structure still plays a role in expressing the rewarding effects of a safety cue. Both d-amphetamine or GABA antagonist administration into the NAs reduces avoidance lever pressing in the presence of a safety cue, demonstrating NAs's role in modulating avoidance behaviors that is reinforced by safety signals (A. B. Fernando, Urcelay, Mar, Dickinson, & Robbins, 2014). The PFC also receives considerable attention for safety learning. Among people with PTSD, their impairment in safety learning has been attributed hypoactivity in the PFC along with hyperactivity in the amygdala (J. P. Christianson et al., 2012). Patients with generalized anxiety disorder also show lower activity in the vmPFC than control patients to an aversive tone during discrimination learning(Laufer, Israeli, & Paz, 2016). The IL within this structure is also necessary

for fear extinction(Kong et al., 2014). However, the role of PFC on safety learning has been controversial. There is a group that found that inactivating both the PL and IL together during safety learning does not impair the safety effect showing that the ventromedial prefrontal cortex is not needed for safety learning(J. P. Christianson et al., 2008). However, there is a different group that found that inactivating the IL and PL separately revealed the IL is necessary for fear and safety discrimination learning(Sangha, Robinson, et al., 2014).The discrepancy could be due to differences in training paradigms. It is possible that inactivation both IL and PL concurrently could have also impair fear expression since the PL and IL have differential roles in fear expression and safety learning, respectively.

Different Than Extinction

In fear extinction, a previously learned CS is no longer followed by the US. As a result, the animals gradually show less conditioned fear responses to the CS. However, this fear reduction in fear extinction have distinct impacts and mechanisms than that of learned safety. Fear extinction only inhibits fear to the once previously conditioned signal. In contrast, learned safety has broader antidepressant and reward effects because it can inhibit innate fear and facilitate reward behaviors(Kong et al., 2014). These two types of learning are mediated by distinct neural circuits.

Electrophysiological data from the BLA have demonstrated the presence of neurons that are responsive to the safety cue, combined fear + safety cue and reward cues in the BLA indicating that the BLA has an overlapping neural circuit encoding safety and reward learning (Sangha et al., 2013), further supporting an old idea that a safety cue has rewarding properties (Rescorla, 1969). As a result, unlike extinction, the

presence of safety cues can elicit approach behaviors and facilitate the acquisition of operant tasks (J. P. Christianson et al., 2012). In addition, the number of BLA neurons that show selective responding to the fear cue normally increase during extinction(Sangha, 2015). However, the proportion of neurons within the BLA that switch to become fear cue responsive does not differ between safety neurons and other neurons during fear extinction(Sangha, 2015). This demonstrates that safety learning and fear extinction likely involve independent circuits within the BLA.

Taking everything together, these findings suggest that dopamine signaling within the BLA should modulate safety-fear-reward cue discrimination. Since there is a high concentration of D1 receptors, but little to no expression of D2 receptors, in the BLA (Abraham et al., 2014; Weiner et al., 1991), the current project focuses on the effect of manipulating D1 receptor activity within the BLA.

Our program's long-term goal is to better understand comorbid disorders arising from emotional dysregulation. Our objective in this study is to identify the contribution of BLA dopamine D1 receptor activity in mediating safety-fear-reward cue discrimination using a rodent model. Our central hypothesis is that dopaminergic D1 receptor activity in the BLA is essential for safety-fear-reward cue discrimination. Our rationale for this research is to guide future research to better target the necessary pathway(s) to manipulate and electrophysiologically monitor. This is essential for linking cellular level changes to behavioral expression. We propose to test our central hypothesis with the following two aims:

Specific Aims

Aim 1. Hypothesis: A) Since dopamine D1 receptor activity in the amygdala, NAc, and hippocampus mediates fear learning(Fadok et al., 2010; Heath et al., 2015), **increasing dopamine D1-receptor activity globally with an agonist should enhance fear learning and impair safety learning. Decreasing D1 receptor activity with an antagonist globally should produce the opposite effect of enhancing safety learning and impairing fear learning.** B) Since dopamine D1 receptor activity in the PFC, NAc, amygdala and hippocampus mediates reward learning(Matthew E. Andrzejewski & Ryals, 2016; Tobin et al., 2013), **increasing dopamine D1 receptor activity globally with an agonist should enhance discriminative reward seeking. Blocking dopamine D1 receptor activity globally with an antagonist should produce the opposite effect of impairing discriminative reward learning.** This will be tested by using subcutaneous administration of a D1 receptor agonist, D1 receptor antagonist or saline 20 minutes prior to each discrimination session where rats are learning about cues signifying safety, fear or reward using the procedure previously published (Sangha et al., 2013; Sangha, Robinson, et al., 2014). In contrast to only administering the drug prior to the last discrimination session, administrating the drug prior to each discrimination session allows us to examine the drug's impact during discrimination acquisition.

Aim 2. Hypothesis: Dopamine D1 receptor activity in the BLA is needed for both fear learning (Heath et al., 2015) and reward learning (Matthew E. Andrzejewski & Ryals, 2016). Dopamine levels in the BLA increase during fear learning (de Oliveira et al., 2011), and BLA neurons contribute to encoding reward prediction error (Wassum & Izquierdo, 2015). **Increasing dopamine D1-receptor activity in the BLA with an agonist should enhance fear learning and impair safety learning, whereas decreasing BLA D1 receptor activity with an antagonist should enhance safety learning and impair fear learning. In addition, increasing dopamine D1 receptor activity in the BLA with an agonist should enhance discriminative reward seeking, while blocking dopamine D1 receptor activity in the BLA with an antagonist should impair discriminative reward learning.** This will be tested by local BLA infusions of a D1 receptor agonist, D1 receptor antagonist or saline 20 minutes prior to each discrimination session where rats are learning about cues signifying safety, fear or reward using the procedure similar to Aim 1.

For both Aims 1 and 2, it is predicted that fear/safety discrimination will be impaired when D1 receptor activity is induced with an agonist. In addition, fear learning and discriminative reward activity is expected to be impaired when D1 receptor activity is blocked with an antagonist in the BLA.

MATERIALS AND METHODS

Subjects

Fifty-four Long Evans male rats (Harlan) weighing 300-350 g were single housed (12h light/dark cycle, lights on 09:00) and handled for 1 week before commencing experiments. All procedures were performed during the light cycle and approved by the Purdue Animal Care and Use Committee. Rats had *ad libitum* access to food and water up until the first training session, when they were restricted to 20 g of food per day for the remainder of the experiment.

Apparatus

Operant chambers were Plexiglas boxes (32 cm length x 25 cm width x 30 cm height) encased in sound-attenuating chambers (Med Associates, ST Albans, VT). 10% liquid sucrose was delivered through a recessed port 2 cm above the floor in the center of one wall. Port entries and exits were monitored through an infrared beam. Two lights (28v, 100mA) located 10.5 cm from floor on either side of the port served as the 20-s continuous light cue. A light (28v, 100 mA) 27cm above the floor on the wall opposite the port provided constant illumination. Auditory cues were delivered via a "tweeter" speaker (ENV-224BM) located 24 cm from the floor on the same wall as the port. Footshocks were delivered through a grid floor via a constant current aversive

stimulator (ENV-414S). A side video camera located on the door of the sound-attenuating cubicle recorded the rat's behavior for offline video analyses.

Surgery

Rats were anesthetized with isoflurane and stereotaxically implanted bilaterally with stainless steel 27- gauge guide cannula dorsal to the BA (AP -2.2mm; ML +/-4.9; DV -7.5). During infusions, 32 gauge needles extended 1 mm beyond the guide cannulas into the BA. Rats were allowed 7 – 10 days to recover in which they had *ad libitum* access to food and water. Stainless steel 32-gauge dummy cannulas were inserted into the guide cannulas between infusions.

Behavioral Training Paradigm

The three cues signifying reward, fear or safety were a 20 s continuous 3 kHz tone (70dB), a 20 s pulsing 11 kHz tone (200 ms on, 200 ms off; 70 dB), or a 20 s continuous light (28V, 100 mA), respectively. The stimuli were not counterbalanced for this study since our previous study did not find significant differences in conditioned freezing among any of the cues (Sangha et al, 2013).

Rats were trained in 3 phases. Phase 1 consisted of a reward session administered on 5 separate days. Each session had 25 paired presentations of a 20s reward cue with a 3 s delivery of 10% sucrose solution (100 μL) into the port accessible to the animals. Sucrose delivery commenced pseudorandomly 10 to 20 s after reward cue onset. The intertrial interval (ITI) was 90 -130 s. Phase 2 consisted of a single habituation session. Animals continued to receive 25 trials of reward cue-sucrose pairings as well as 5 additional trials each of the fear cue presented alone and safety cue presented alone (ITI, 90-130 s). This procedure allows the animals to

habituate and reduce their baseline freezing to the novel cues but does not contain enough presentations to produce latent inhibition. Phase 3 consisted of four sessions of discrimination training. For each session, delivered on separate days, rats received: 15 trials of reward cue-sucrose pairings, 4 trials of fear cue-footshock pairings (20 s cue + 0.5 s, 0.45 mA footshock at cue offset), 15 trials of simultaneous presentation of the 20 s fear and safety cues without footshock, and 10 trials of the 20 s safety cue presented alone without footshock (total 44 trials, ITI 100 – 140 s). Inclusion of trials where the safety cue was presented alone was to provide the animal with additional trials with a safety cue- no shock contingency and to assess if freezing developed to the safety cue.

Systemic Injections

Systemic s.c. injections of a D1 receptor agonist (10mg/kg SKF-38393)(Doty et al., 1998; Inoue, Izumi, Maki, Muraki, & Koyama, 2000), antagonist (3. 33µg/kg SCH-23390) (Sciascia, Mendoza, & Chaudhri, 2014)or saline were administered 20 m prior to each DC session. Previous study have shown that systemic administration of SKF-38393 can increase and systemic administration of SCH-23390 can decrease Ach release in striatal neurons in as little as 20 minutes, respectively (Johnson & Bruno, 1995). This provides support that our 20 minute wait time after systemic injection should be sufficient to allow both the D1 receptor agonist and antagonist to reach the brain. To acclimate the animals to the injection procedure, all rats also received saline injections 20 minutes prior to the last reward training and habituation training.

BLA Infusions

D1 dopamine receptor agonist SKF38393 was dissolved in 0.9% sodium chloride with concentrations of 1 µg/0.5µL (Zarrindast, Rezayof, Sahraei,

Haeri-Rohani, & Rassouli, 2003). The antagonist SCH 23390 was dissolved in 0.9% sodium chloride with concentrations of 0.25 μg/0.5μL (Hikind & Maroun, 2008). Twenty minutes prior to each DC session, 0.5 μL of the mixture was infused (0.25 μL/s) into the BLA bilaterally. The injectors were left in place for 2 min post-infusion to allow for drug diffusion. A separate group of animals received saline infusions instead. In order to habituate animals to the infusion procedure, all animals received sham infusions 20 min prior to the last reward session and habituation session.

Histology

Rats were deeply anesthetized with sodium pentobarbital, and then perfused with PBS followed by 10 % formalin. Tissues were then post-fixed in 30% sucrose formalin and sectioned at 50 μm with a cryostat. Sections were then plated on glass slides and stained with cresyl violet. Slides were examined under a light microscope to verify placements. 27 out of 48 rats had verified bilateral cannula placements in the BLA and only these subjects were included in the analyses.

Data Analysis

Fear behavior was assessed offline from videos by measuring freezing, defined as complete immobility with the exception of respiratory movements, which is an innate defensive behavior (Blanchard & Blanchard, 1969; Fendt & Fanselow, 1999). The total time spent freezing during the presentation of each 20 s cue was quantified. Measuring the total time the animal spent inside the reward port and at the entrance of the port with nose positioned at port entrance to assess reward behavior. We also calculated a fear discrimination ratio (% Freezing to the fear cue/ (% Freezing to the reward cue + % Freezing to the safety cue + % Freezing to the fear + safety cue)) and

reward discrimination ratio (% Port to the reward cue / (% Port to the fear cue + % Port to the safety cue + % Port to the fear + safety cue)) for each animal and each session. Six individuals blind to drug treatment and cannula placement performed the behavioral scoring. Pearson's correlations of freezing and reward behavior values between scorers were greater than $r = 0.80$. Behavioral data were analyzed with two way ANOVAs followed by Dunnett's post-hoc tests with GraphPad Prism. In our discrimination paradigm, saline animals typically start to show evidence of safety learning during DC3, with maximal discrimination among the different cues evidient in DC4 (Sangha, Robinson, et al., 2014). Thus, we focused on testing for behavioral differences in DC4. In contrast, using repeated measures among all four DC sessions increases the number of unnecessary comparisons and compromising the likelihood of detecting the learning related effect in the last session. We anticipated that the animals will show more freezing to the fear cue than all other cues, and will show more port activity to the reward cue than all other cues. In order to reduce numbers of unnecessary comparisons, we used Dunnett's test instead of Tukey to selectively compare the activity to a specific cue against all other cues.

RESULTS

Locomotor Effects of the D1 Receptor Agonist and Antagonist

To examine possible locomotor impairment from systemic or local administration of D1 receptor agonist, D1 receptor antagonist or saline, we analyzed the motion activity in the first and last five minutes of each session. The five minutes windows were chosen because the number five is a prime number and its multiple is easy to work with for potential normalizing or comparison. Analyzing the last five minutes allows us to verify the duration of action of the drug throughout the entire training session. If a drug influence locomotor activity in the first 5 minutes, we expect it should have the same influence in the last 5 minutes.

Systemic Injection

A two way repeated measures ANOVA (time by treatment) was performed on motion activity during the first and last five minutes of each DC session to look at the effect of drug on locomotor activity. There was a significant main effect of time across all DC sessions (DC1: $F(1, 24) = 80.98$, $p < 0.0001$; DC2: $F(1, 24) = 79.05$, $p < 0.0001$); DC3: $F(1, 24) = 74.32$, $p < 0.0001$; DC4: $F(1, 24) = 76.83$, $p < 0.0001$). Post hoc comparison with Bonferroni correction showed that animals from each treatment condition exhibited more locomotor activity in the first five minutes than the last five minutes ($p < 0.05$). Since this effect was also seen in the saline group, this effect is most likely due to reduced exploration of the conditioning chamber over time. There

was also a significant main effect of treatment in DC4 ($F(2, 24) = 4.36$, $p < 0.05$), but not DC1, 2 or 3. Post hoc comparison with Bonferroni correction showed that saline animals had more locomotor activity than antagonist animals in the last 5 minutes ($p < 0.05$) for only DC4. This reduction in locomotor activity from the antagonist could lead to the artificial increase in freezing score to all cues toward the end of DC4. Taken together, these data indicate that systemic administration of D1 receptor agonist or antagonist does not affect overall locomotor activity.

BLA Infusion

A two way repeated measures ANOVA (time by treatment) was performed on motion activity during the first and last five minutes of each DC session to look at the effect of drug on locomotor activity. There was a significant main effect of time across all DC sessions (DC1: $F(1, 24) = 44.67$, $p < 0.0001$; DC2: $F(1, 24) = 55.1$, $p < 0.0001$); DC3: $F(1, 24) = 80.41$, $p < 0.0001$; DC4: $F(1, 24) = 138.1$, $p < 0.0001$). Post hoc comparison with Bonferroni correction showed that animals from each treatment condition exhibited more locomotor activity in the first five minutes than the last five minutes ($p<0.05$). Similar to what was seen with systemic injections, since this effect was also seen in the saline group, this effect is most likely due to reduced exploration of the conditioning chamber over time. No main effects of treatment were found. These data indicate that BLA infusion of D1 receptor agonist or antagonist has no effect on overall locomotor activity.

Systemic Injection of D1 Receptor Agonist or Antagonist Blocks Fear and Safety Discrimination

Twenty minutes prior to the start of each discrimination training session, rats received a systemic injection of a D1 receptor agonist (10mg/kg SKF-38393; $N = 8$), antagonist (3. 33µg/kg SCH-23390; $N = 7$) or saline ($N = 12$). During discrimination training, rats were presented with cues signifying fear, reward, safety and the combined fear + safety. The percent time spent freezing for each cue was calculated (Figure 2), as well as the percent time spent in the port in response to each cue (Figure 3). Since asymptotic level of learning occurred during the last session of discrimination training, current analysis will focus on DC4. Two-way ANOVAs on percent time spent freezing for each cue during DC4 (Figure 2) showed significant cue by treatment interactions ($F(6, 72) = 2.54$, $p < 0.05$). A main effect of cue was found for DC4($F(3, 72) = 299$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the fear cue showed that saline treated animals displayed significantly more freezing to the fear cue than all the other cues ($p < 0.05$ each), indicating good fear discrimination. Animals with systemic injections of the D1 receptor agonist or antagonist showed more freezing to the fear cue than the reward cue and safety cues (Dunnett's multiple comparison to fear cue, $p < 0.05$ each). However, neither group showed significantly more freezing to the fear cue than the combined fear + safety cue ($p > 0.05$). This indicates that systemic injections of a D1 receptor agonist or antagonist impair fear suppression in the presence of the safety cue. A main effect of treatment was also found for DC4 ($F(2, 24) = 14.95$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the saline group showed that D1 receptor agonist treated group had higher levels of freezing to the reward, safety, and

fear + safety cue than saline animals. Even though we previously reported that antagonist animals had reduced locomotor activity toward the end of DC4, but current results suggested that the reduced locomotor activity did not appear to increase freezing scores in the antagonist group.

To further assess fear discrimination behavior, we also calculated discriminative fear ratios for a broader comparison of freezing levels to the fear cue relative to the sum of freezing to all other cues. The ratios were calculated for each animal then averaged (Figure 2). A fear discrimination ratio larger than one indicates more freezing to the fear cue than all other cues and would be indicative of good discrimination. One-way ANOVAs on the fear discrimination ratios during DC4 showed a significant effect of treatment ($F(2, 24) = 7.21$, $p < 0.01$). *Post hoc* Dunnett's multiple comparisons to the saline group showed that fear discrimination ratios were significantly lower in the D1 receptor agonist treated animals ($p < 0.05$) but not the D1 receptor antagonist treated animals ($p > 0.05$).

We have used two methods to quantify fear discrimination behavior: 1) averaged percent time freezing to the fear cue versus the combined fear+safety cue and, 2) within animal fear discrimination ratios of freezing to the fear cue compared to all other cues. Fear discrimination, as assessed by comparing averaged percent time freezing relative to the combined fear+ safety cue, was impaired by systemic injections of either the D1 receptor antagonist and agonist. In addition, systemic injections of a D1 receptor agonist significantly lowered the fear discrimination ratio. Taken together, both the agonist and antagonist impair fear discrimination behavior.

For the same DC4 session, discriminatory reward seeking was also analyzed. Two-way ANOVAs on percent time spent in the port for each cue during DC4 (Figure 3) showed a significant cue by treatment interaction ($F(6, 72) = 7.58$, $p < 0.001$). A main effect of cue was found for DC4 ($F(3, 72) = 38.99$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the reward cue showed that the saline group showed more port activity to the reward cues than all the other cues ($p < 0.05$ each), which was also seen in the D1 receptor antagonist group ($p < 0.05$ each). The D1 receptor agonist group did not show more port activity during the reward cue than all other cues ($p > 0.05$). These data indicate that systemic injection of a D1 receptor agonist decreased discriminative port activity. A main effect of treatment was also found for DC4 ($F(2, 24) = 7.52$, $p < 0.01$). *Post hoc* Dunnett's multiple comparisons showed that saline animals had higher port activity to the reward cue than D1 receptor agonist treated animals ($p < 0.05$).

Reward discrimination ratios were calculated using the ratio between port activities in response to the reward cue versus the sum of port activity in response to the other cues for each animal (Figure 3). One-way ANOVAs on reward discrimination ratios for each DC session showed a significant treatment effect during DC4 ($F(2, 24) = 7.77$, $p < 0.01$). *Post hoc* Dunnett's multiple comparisons to the saline group showed significantly lower reward discrimination ratios in the D1 receptor agonist treated animals ($p < 0.05$) but not the D1 receptor antagonist treated animals ($p > 0.05$). These data indicate that systemic injection of a D1 receptor agonist, but not antagonist, impairs discriminative reward seeking.

Systemic Injection of D1 Receptor Agonist or Antagonist During DC1-3

Systemic Freezing

There were significant cue by treatment interactions for sessions DC 2-3 (DC2: $F(6, 72) = 2.58$, $p < 0.05$; DC3: $F(6, 72) = 3.40$, $p < 0.01$) , but not DC 1 ($F(6, 72) = 2.07$, $p = 0.07$). A main effect of cue was found for DC1-3 sessions (DC1: $F(3, 72) = 40.38$, $p < 0.0001$; DC2: $F(3, 72) = 187.9$, $p < 0.0001$; DC3: $F(3, 72) = 249.1$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the fear cue showed that saline treated animals displayed significantly more freezing to the fear cue than all the other cues ($p < 0.05$ each) during sessions DC2, and DC3. Animals with systemic injections of the D1 receptor agonist or antagonist showed more freezing to the fear cue than the reward cue and safety cues (Dunnett's multiple comparison to fear cue, $p < 0.05$ each) during sessions DC2 and DC3. However, neither group showed significantly more freezing to the fear cue than the combined fear + safety cue ($p > 0.05$) during sessions DC1, DC2, or DC3. A main effect of treatment was also found for DC1, 2 and 3. (DC1: $F(2, 24) = 6.30$, $p < 0.01$, DC2: $F(2, 24) = 8.11$, $p < 0.01$, DC3: $F(2, 24) = 12.36$, $p < 0.001$). *Post hoc* Dunnett's multiple comparisons to the saline group showed higher levels of freezing to the fear cue in the saline group than the antagonist group during DC1, DC2, and DC3. Saline animals showed lower levels of freezing to the reward and safety cues than the D1 receptor agonist treated group during sessions DC2 and DC3. One-way ANOVAs on the fear discrimination ratios during each DC session showed a significant effect of treatment during DC2 (DC2: $F(2, 24) = 4.72$, $p < 0.05$), but not DC 1 or 3 (DC1: $F(2, 24) = 0.39$, $p = 0.68$; DC3: $F(2, 24) = 2.40$, $p = 0.11$). For DC2, *post hoc* Dunnett's multiple comparisons to the saline group showed

fear discrimination ratios were significantly lower in the D1 receptor agonist treated animals ($p < 0.05$) but not the D1 receptor antagonist treated animals ($p > 0.05$).

Systemic Port

Two-way ANOVAs on percent time spent in the port for each cue during each DC session (Figure 3) showed a significant cue by treatment interaction for DC1-3 (DC1: $F(6, 72) = 3.11$, $p < 0.01$; DC2: $F(6, 72) = 2.56$, $p < 0.05$; DC3: $F(6, 72) = 7.93$, $p < 0.001$). A main effect of cue was found for DC1-3 (DC1: $F(3, 72) = 46.95$, $p < 0.0001$; DC2: $F(3, 72) = 38.41$, $p < 0.0001$; DC3: $F(3, 72) = 22.29$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the reward cue showed that the saline group showed more port activity to the reward cues than all the other cues ($p < 0.05$ each) during DC1-3.The D1 receptor antagonist groups showed more port activity to the reward cues than all the other cues during DC1 and 2 ($p < 0.05$ each), but not DC3 ($p > 0.05$). The D1 receptor agonist group did show more port activity during the reward cue than all other cues ($p < 0.05$) during DC1, but not during DC2-3 ($p > 0.05$). A main effect of treatment was also found for DC1-3 (DC1: $F(2, 24) = 4.56$, $p < 0.05$; DC2: $F(2, 24) = 7.48$, $p < 0.01$; DC3: $F(2, 24) = 19.87$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the saline group showed that saline animals had higher port activity to the reward cue than D1 receptor agonist treated animals ($p < 0.05$) during sessions DC1, DC2, and DC3. One-way ANOVAs on reward discrimination ratios for each DC session did not show significant treatment effect during any DC1-3 (DC1: $F(2,24) = 1.83$, $p = 0.18$; DC2: $F(2, 24) = 0.83$, $p = 0.45$; DC3: $F(2, 24) = 1.91$, $p = 0.17$).

Taken together systemic injections of the D1 receptor agonist, SKF-38393, and antagonist, SCH-23390, both impair suppression of freezing to the fear cue when in the presence of the safety cue (Figure 2). The D1 receptor antagonist did not significantly impair reward seeking during the reward cue when compared to the other cues. The D1 receptor agonist, however, did appear to significantly suppress reward seeking during all cues (Figure 3). Since D1 receptor agonist animals did show a high level of discriminative reward seeking behaviors and local motor activities during the first session of discrimination, the observed impairment over subsequent days could be due occlusion of reward seeking behavior. To minimize the negative influence of the non-specific effects of the drugs on behavioral expression, the same drugs were infused directly into the BLA in Experiment 2.

BLA Infusion of a D1 Receptor Agonist, but not Antagonist, Impairs Discriminative Fear Learning

Dopamine signaling within the BLA has been implicated in fear learning, fear extinction and reward learning. The systemic administration of D1 receptor agonist or antagonist could be producing its impairment in fear suppression and reward seeking by exerting its effects through the BLA. To localize the effect of D1 receptor activity, twenty minutes prior to the start of each DC session, 0.5 µL of D1 receptor agonist (1 µg/0.5µL SKF-38393; $N = 9$), antagonist (0.25 µg/0.5µL SCH-23390; $N = 8$) or saline ($N = 10$) were infused directly into the BLA bilaterally. Only animals with confirmed bilateral placements of cannula tips in the BLA were included in the analyses (27 of 48 animals; Figure 4). During discrimination training, rats were presented with cues signifying fear, reward, safety and the combined fear + safety. The percent time spent

freezing for each cue was calculated (Figure 5), as well as the percent time spent in the port in response to each cue (Figure 6).

Since asymptotic level of learning occurred during the last session of discrimination training, current analysis will focus on DC4. Two-way ANOVAs on percent time spent freezing for each cue during DC4 (Figure 5) showed significant main effect of cue ($F(3, 72) = 102.2$, $p < 0.001$). *Post hoc* Dunnett's multiple comparisons to the fear cue showed that the saline group froze significantly more to the fear cue in comparison to the other cues ($p < 0.05$). Similarly, the D1 receptor antagonist group showed more freezing to the fear cue in comparison to the other cues ($p < 0.05$). The D1 receptor agonist group, however, only showed more freezing to the fear cue compared to the reward and safety cues, and not the fear+safety cue. Together this indicates that BLA infusion of a D1 receptor agonist, but not antagonist, impairs fear suppression in the presence of safety cue. A main effect of treatment was also found for DC4 ($F(2, 24) = 4.46$, $p < 0.05$). *Post hoc* Dunnett's multiple comparisons to the saline group showed that D1 receptor agonist treated animals had higher levels of freezing to the reward cue and to the safety cue. One way ANOVAs on fear discrimination ratios for DC4 showed a significant main effect of treatment ($F(2, 24) = 5.70$, $p < 0.01$)). *Post hoc* Dunnett's multiple comparisons to the saline group showed that saline animals had higher fear discrimination ratios than agonist animals but not antagonist animals in DC4. This indicates that infusion of the agonist directly into the BLA impairs discriminative freezing to the fear cue relative to the other cues.

A two way ANOVA on percentage of time spent in the port (Figure 6) showed a significant main effect of cue for DC4 ($F(3, 72) = 20.86$, $p < 0.0001$). *Post hoc*

Dunnett's multiple comparisons to the reward cue showed that the saline and antagonist groups exhibited more port activity to the reward cue in comparison to the other cues. The agonist group also showed more port activity to the reward cue than the fear + safety and safety cues, but not the fear cue. One way ANOVAs on reward discrimination ratios for each DC session showed no significant main effects of treatment during DC4 ($F(2, 24) = 1.89$, $p < 0.17$). Overall, this indicates that infusing an agonist or antagonist into the BLA does not impair discriminative reward behavior.

BLA infusion of D1 Receptor Agonist or Antagonist During DC1-3

Infusion Freezing

Two-way ANOVAs on percent time spent freezing for each cue during DC 1- 3 (Figure 5) showed significant main effect of cue (DC1: $F(3, 72) = 14.01$, $p < 0.0001$; DC2: $F(3, 72) = 45.44$, $p < 0.0001$; DC3: $F(3, 72) = 55.17$). *Post hoc* Dunnett's multiple comparisons to the fear cue showed that the saline group froze significantly more to the fear cue in comparison to the other cues ($p < 0.05$) during sessions DC1 and DC3. A main effect of treatment was also found for DC1-3 (DC1: $F(2, 24) = 4.75$, $p < 0.05$; DC2: $F(2, 24) = 7.09$, $p < 0.01$; DC3: $F(2, 24) = 3.61$, $p < 0.05$). Saline treated animals showed more freezing to the fear cue than the reward cue and safety cue during DC2. The D1 receptor antagonist group showed more freezing to the fear cue in comparison to the other cues ($p < 0.05$) during DC2. This group also showed more freezing to the fear cue than the reward cue and safety cue, but not the combined fear + safety cue during DC3. The D1 receptor agonist group showed more freezing to the fear cue than the reward cue and safety cue during DC1, DC2 and DC3. This group also showed more freezing to the fear cue than the combined fear + safety cue during

DC3, but not DC1 and DC2. *Post hoc* Dunnett's multiple comparisons to the saline group showed that D1 receptor agonist treated animals had higher levels of freezing to the combined fear + safety cue than the saline group during DC1 and DC2. In comparison to the saline group, the agonist group also showed higher levels of freezing to the fear cue during DC2 and reward cue during DC3. The D1 receptor antagonist group, on the other hand, showed less freezing to the fear cue than saline animals during DC1. One way ANOVAs on fear discrimination ratios for each DC session showed a significant main effect of treatment for DC2 ($F(2, 24) = 3.45$, $p < 0.05$) but not DC1 and DC3 (DC1: $F(2, 24) = 0.41$, $p = 0.67$; DC3: $F(2, 24) = 2.73$, $p = 0.09$).

Infusion Port

A two way ANOVA on percentage of time spent in the port (Figure 6) showed a significant main effect of cue for DC1-3 (DC1: $F(3, 72) = 48.08$, $p < 0.001$; DC2: $F(3, 72) = 41.92$, $p < 0.001$; DC3: $F(3, 72) = 16.24$, $p < 0.0001$). *Post hoc* Dunnett's multiple comparisons to the reward cue showed that the saline and antagonist groups exhibited more port activity to the reward cue in comparison to the other cues during DC1, DC2 and DC3. The agonist group also showed more port activity to the reward cue than the other cues during DC1 and DC2. This group also showed more port activity to the reward cue than the fear + safety and safety cues, but not the fear cue, during DC3. One way ANOVAs on reward discrimination ratios for each DC session showed no significant main effects of treatment during DC1-3 (DC1: $F(2, 24) = 0.81$, $p = 0.46$; DC2: $F(2, 24) = 0.67$, $p = 0.52$; DC3: $F(2, 24) = 0.31$, $p = 0.74$).

Taken together, the impairment in discriminative fear that was seen during systemic injection of the D1 receptor agonist, SKF-38393, was replicated when the

agonist was infused directly into the BLA. However, the D1 receptor antagonist, SCH-23390, only had an effect on fear and safety discrimination during systemic administration and not during direct BLA infusion. This indicates that the fear discrimination impairment seen in the systemic D1 receptor antagonist group could be having its effect on areas outside the BLA. In addition, the D1 receptor agonist only had an effect on discriminative reward seeking when it was administered systemically but not when it was directly infused into the BLA. This indicates that dopamine D1 receptor activity in the BLA is not critical for discriminative reward behavior.

Correlation of Movement With Fear Learning

To examine the possible relationship between contextual freezing and cue induced conditioned freezing, correlation coefficient r was calculated for each treatment condition to measure the strength of association between the first five minute of locomotor activities and freezing to the fear cue in DC4. No significant correlation was found for any of the treatment conditions in either systemic or BLA infusion conditions in DC4.

Comparing Behavior Between the First Half and Second Half of Each Discrimination Session

As an additional way to verify that the effect of the drugs last throughout the entire training session, we also compare freezing level and port activity to each of the cues between the first half and second half of each session. Dividing the time into first half and second half of the session allow us to look at potential recovery of learning as the drug effect reduces. Post hoc tests with Bonferroni corrections were used to compare the differences in freezing or port levels among cues between the first half and

the second half of each session. Post hoc Dunnett's test was used to compare the differences in percent time spent freezing among different cues against percent time freezing to the fear cue. If there is an increase in generalized freezing to all cues from the first half to the second half of the session, the main effect of time should be statistically significant. In contrast, if there is a selective and discriminative increase in freezing to the fear cue relative to other cues, the time by cue interaction should be statistically significant. Post hoc Dunnett's test was also used to compare the differences in percent time spent on port activity among different cues against percent time spent on port activity to the reward cue. Similar to freezing mentioned above, increased in generalized port activity to all cues from first half to the second half of the session should result in the main effect of time, and discriminative increase in port activity to the reward cue relative to the other cues should lead to time by cue interaction.

Discriminative Freezing After Systemic Injection

A two way repeated measures ANOVA (time by cue) was performed on percent time spent freezing for each treatment condition during each DC session. The results are summarized in Table 2. Saline animals showed a significant time by cue interaction during DC1 and a significant main effect of time during DC1 and 2. These results reflect within session learning because saline animals were not under drug influence. They also showed more freezing to the fear cue in the second half than the first half of the DC1. During DC3 and 4, saline animals no longer show significant time by cue interactions or significant main effects of time, indicating that the magnitude of freezing to the fear cue did not change during the first half to the second

half of DC3 and DC4. Both agonist and antagonist animals showed significant time by cue interactions and main effects of cues during DC1 indicating that changes in freezing occurred within DC1. However, no significant time by cue interactions or main effects of time during DC2 to 4 for either the agonist or antagonist animals were observed, indicating that no detectable changes in freezing occurred within those subsequent DC sessions.

Discriminative Reward Seeking After Systemic Injection

The same analyses done for percent time in port and the results are summarized in Table 3. There was a significant main effect of time and time by cue interaction during DC1 for saline animals, and DC1 and 2 for antagonist animals. Again, these effects reflect within session learning during earlier DC sessions, but not in DC3 or 4. Agonist animals showed significant cue by time interaction during DC2, and a main effect of time during DC3. These indicate that the systemic administration of agonist occludes the behavioral expression of port behavior. Agonist animals showed more port activity to the reward cues than to some of the other cues only during the first half of DC1 and DC2.

Discriminative Freezing After BLA Infusion

A two way repeated measures ANOVA (time by cue) was performed on percent time spent freezing for each treatment condition during each DC session. The results are summarized in Table 4. Saline animals showed significant cue by treatment interaction during DC3 and 4, but not DC1 and 2. This again reflects within session learning. Antagonist animals showed a significant cue by treatment interaction during DC3 only. These indicate that there was some learning related changes during the later

DC sessions. Agonist animals had a significant main effect of time in DC2 only. This also reflects some within session learning in DC2.

Discriminative Reward Seeking After BLA Infusion

The same analyses were done for percent time in port and the results are summarized in Table 5. No significant main effects of time or time by cue interactions were detected for saline animals in any DC session. A main effect of time was detected in antagonist animals during DC4. A significant main effect of time and time by cue interaction was detected in agonist animals during DC2 only.

Summary

If the administered drug has a short lifespan, we expect to see significant cue by time interaction with more freezing or port activity to the respective cues in the second half than the first half of the session. An overall increase in freezing or port activity from the first half of the session to the second half would be reflected as a main effect of time, and a selective increase in freezing or port activity to the conditioned cue from the first half to the second half would be reflected as a time by cue interaction. Animals with systemic injections of the D1 receptor agonist or antagonist showed more freezing to the fear cue than the reward cue and safety cues (Dunnett's multiple comparison to fear cue, $p<0.05$ each) during sessions DC2, DC3 and DC4. However, neither group showed significantly more freezing to the fear cue than the combined fear + safety cue ($p>0.05$) during sessions DC1, DC2, DC3, or DC4. These indicate an impairment in fear suppression during DC2, 3 and 4. Since no significant main effect of time or time by cue interaction was detected during those sessions for antagonist and agonist animals, there were no within session changes. This suggests that the effects of

the systemically administered drugs last throughout the entire two hour training session. During BLA infusion, the D1 receptor agonist group showed more freezing to the fear cue than the reward cue and safety cue during DC1, DC2, DC3 and DC4. This group also showed more freezing to the fear cue than the combined fear + safety cue during DC3, but not DC1, DC2 and DC4. Since no significant main effects of time or time by cue interactions were detected for these animals during DC3 or 4, the effect of BLA infused agonist persisted throughout the entire two hour training session. On the other hand, BLA infusion of the antagonist did not produce any impairment in conditioned freezing or reward seeking. The current within session analysis only showed time by cue interaction in freezing activity during DC3, but not in any other sessions. Overall, there are no evidence of the BLA antagonist effect diminishing in the second half of the two hour training session.

DISCUSSION

To investigate BLA dopamine D1 receptor activity in mediating fear-reward-safety discrimination learning, we hypothesized that increasing dopamine D1 receptor activity in the BLA should impair fear/safety discrimination learning, enhance fear learning, and enhance discriminative reward seeking. Decreasing dopamine D1 receptor activity in the BLA should impair fear learning, enhance fear/safety discrimination learning and impair discriminative reward seeking. Animals that received a systemic injection or BLA infusion of saline showed good discrimination learning: they froze more to the fear cue and spent more time at the port during the reward cue compared to the other cues. Since saline animals showed more freezing to the fear cue than the combined fear + safety cue, they demonstrated learned safety by suppressing fear in the presence of the safety signal. These are consistent with previous findings (Sangha et al., 2013; Sangha, Greba, Robinson, Ballendine, & Howland, 2014; Sangha, Robinson, et al., 2014). In addition, the systemic injection of a D1 receptor agonist or antagonist blocked fear/safety discrimination learning. Systemic administration of the agonist, but not antagonist, also impaired the discriminative fear and reward ratios. When the agonist was infused directly into the BLA, we replicated the impairments in fear/safety discrimination and the discriminative fear ratio, but not the impairment in discriminative reward seeking.

BLA infusion of the antagonist had no effect on the fear/safety discrimination, fear discrimination ratio or discriminative reward seeking. These data support our hypothesis that increasing dopamine D1 receptor activity in the BLA impairs fear and safety discrimination behavior. However, our data does not support the hypothesis that decreasing dopamine D1 receptor activity impairs discriminative reward seeking behavior.

Taken together, these data suggest that dopamine D1 receptor activity in the BLA is not necessary for discriminative reward seeking, safety learning or discriminative fear learning. Blocking D1 receptor activity in the BLA with a D1 receptor antagonist did not impair discriminative reward seeking, fear and safety discrimination or discriminative fear learning. However, increasing D1 receptor activity, either systemically or directly in the BLA, impairs fear and safety discrimination. We found a similar impairment in the discriminative fear ratio when comparing freezing behavior to the fear cue against freezing behavior to all other cues regardless if the agonist was administered systemically or infused locally in the BLA. Animals that received systemic or BLA administration of the agonist were still able to show more freezing to the fear cue than the reward and safety cues, demonstrating some level of fear discrimination. However, these animals did not show increased freezing to the fear cue relative to the fear+safety cue, demonstrating a selective impairment in suppressing freezing to the fear cue in the presence of the safety cue. This impairment in fear suppression may be due to increased activity in the amygdala through D1 receptor activation. Since the amygdala typically shows a higher CS evoked field potential to a fear CS compared to a safety CS (Rogan et al., 2005), the

stimulation of D1 receptors may prevent the necessary decrease in amygdala activity during safety learning, leading to behavioral impairments in safety but not fear behavior. Increasing D2 receptor activity in the VTA blocks learning related increases in dopamine levels within the BLA during fear conditioning (de Oliveira et al., 2011), implying that BLA dopamine levels are mediated by the VTA.

The impairment in fear and safety discrimination with a systemic D1 receptor antagonist injection was due to impairment in safety learning since animals were still able to showing discriminative fear with respect to both the reward cue and safety cue during discrimination training session two, three and four. D1 receptor antagonist systemic drug action could be acting through the PFC to produce impairment in safety discrimination. The IL is necessary for fear and safety discrimination(Sangha, Robinson, et al., 2014) and it receives dopaminergic projection from the VTA (Abraham et al., 2014) as well as projecting directly to the BLA (Vertes, 2004). D1 receptor activity in the IL is necessary for fear extinction consolidation(Abraham et al., 2014). The systemic administration of the D1 receptor antagonist could produce receptor action in the IL leading to the impairment we observed in safety discrimination.

For discriminative reward behavior, it appears that D1 receptor activity in the BLA is not needed. Neither systemic nor BLA administration of the D1 receptor antagonist had an effect on the reward discrimination ratio. The impairment we observed in discriminative reward seeking with the systemically administered D1 receptor agonist is likely due to impairment in behavioral expression. These animals showed discriminative port activity similar to saline and antagonist treated animals

during the first discrimination training session. Then, all port activity diminished over each subsequent session. This impairment in discriminative reward seeking is not due to D1 receptor action in the BLA because BLA infusion of the D1 receptor agonist had no effect on the discriminative reward ratio. The impairment could however be due to receptor action in the NAc. D1 receptor activity in the NAc is necessary for consumption behavior (Richard & Berridge, 2011) and the reinstatement of drug seeking during stress (Tobin et al., 2013). Dopamine levels in the NAc also gradually increase during different phases of operant behavior that lead to reward outcome(A. L. Collins et al., 2016). This change in dopamine levels is influenced by the BLA and it is D1 receptor activity dependent (Stuber et al., 2011). Activation of this pathway induces reward seeking (Stuber et al., 2011) and inactivation of BLA using muscimoml/baclofen mixture impairs approach behavior to the port (Jones et al., 2010). In light of these findings, it is possible that increasing D1 receptor activity in the NAc via a systemically administered D1 receptor agonist during reward seeking may be reinforcing on its own and may occlude reward seeking behavioral expression.

Our current study indicates that increasing dopamine D1 receptor activity in the BLA may be a potential mechanism that leads to the impairment in fear and safety discrimination seen with PTSD patients. Since the VTA is the primary source of dopamine for the BLA, the VTA might be providing safety information to the BLA. The alteration in dopamine release in the VTA-BLA pathway may contribute to the impairment in fear/safety discrimination in people with PTSD. Enhanced dopamine release may occlude the necessary phasic increase in dopamine release in the VTA-BLA pathway during fear learning whereas decreased dopamine release may occlude

the necessary phasic decrease in dopamine levels in the VTA-BLA pathway during safety learning. This would indicate that the VTA-BLA dopamine signaling pathways may be potential therapeutic target sites for treating PTSD.

Manipulating dopamine receptor activity has been reported to affect locomotion activity(Pezze, Marshall, & Cassaday, 2016; Tran et al., 2005). To avoid this problem, we used dosages that have been published by other labs using the same method of administration as the current study. In addition, examining the first five minutes of locomotor activity prior of each training session did not show impairment in locomotion activity for any treatment conditions.

In conclusion, we have identified that increasing D1 receptor activity in the BLA impairs fear/safety discrimination. Currently, it is unclear if inactivating the dopaminergic pathway from the VTA to BLA drives safety behavior. It is also unclear if increased dopamine D1 receptor activity in the NAc interferes with reward seeking. Future studies will use a retro-DREADD approach to activate the VTA to BLA dopamine pathway during fear, safety and reward discrimination to assess if the same impairment in fear/safety discrimination is observed. Future studies can also use retro-DREADDs to activate the BLA to NAc pathway during fear, safety and reward discrimination to investigate if it impairs reward seeking. An impairment would indicate the decrease in reward seeking seen during systemically administered D1 agonist is being mediated by the BLA-NAc pathway.

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APPENDICES

Appendix A

Table 1

Predictions From Specific Aims 1 and 2

Predictions for Specific Aim 1	Fear/Safety Discrimination	Discriminative Fear	Discriminative Reward
Systemic Saline	Yes	Yes	Yes
Systemic Agonist	No	Yes	Yes
Systemic Antagonist	Yes	No	No

Predictions for Specific Aim 2	Fear/Safety Discrimination	Discriminative Fear	Discriminative Reward
BLA Saline	Yes	Yes	Yes
BLA Agonist	No	Yes	Yes
BLA Antagonist	Yes	No	No

Table 2

Comparison of Freezing Percentage to Each of the Cues Between the First Half and Second Half of Each DC Session for Animals That Received Systemic Administration of D1 Receptor Agonist, D1 Receptor Antagonist, or Saline

Systemic Freeze		Saline		Antagonist		Agonist	
DC1	Interaction	**F (3, 44) = 25.09**	**P<0.0001**	**F (3, 24) = 4.632**	**P=0.0108**	**F (3, 28) = 5.512**	**P=0.0042**
	Main effect time	**F (1, 44) = 51.6**	**P<0.0001**	**F (1, 24) = 12.85**	**P=0.0015**	**F (1, 28) = 46.01**	**P<0.0001**
	Main effect cue	**F (3, 44) = 17.03**	**P<0.0001**	**F (3, 24) = 5.788**	**P=0.0040**	**F (3, 28) = 5.81**	**P=0.0032**
	Post hoc Bonferroni	First half < Second half: Fear, Fear + Safety		First half < Second half: Fear		First half < Second half: Fear, Safety	
	Post hoc Dunnett	First half: Fear < Fear + Safety . Second half: Fear > Reward, Safety		First half: N/A . Second half: Fear > Reward, Safety		First half: N/A . Second half: Fear > Reward, Safety	
DC2	Interaction	F (3, 44) = 1.291	P=0.2892	F (3, 24) = 1.755	P=0.1826	F (3, 28) = 2.537	P=0.0769
	Main effect time	**F (1, 44) = 5.777**	**P=0.0205**	F (1, 24) = 0.6337	P=0.4338	F (1, 28) = 3.844	P=0.0599
	Main effect cue	**F (3, 44) = 59.71**	**P<0.0001**	**F (3, 24) = 40.06**	**P<0.0001**	**F (3, 28) = 29**	**P<0.0001**
	Post hoc Bonferroni	First half < Second half: Fear		N/A		N/A	
	Post hoc Dunnett	First half: Fear >Reward, Safety . Second half: Fear > Reward, Fear +safety, Safety		First half: Fear > Reward, Fear +safety, Safety . Second half: Fear > Reward, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Safety	
DC3	Interaction	F (3, 44) = 1.195	P=0.3227	F (3, 24) = 0.5976	P=0.6228	F (3, 28) = 0.7229	P=0.5468
	Main effect time	F (1, 44) = 0.3349	P=0.5657	F (1, 24) = 0.5021	P=0.4854	F (1, 28) = 0.1987	P=0.6592
	Main effect cue	**F (3, 44) = 121.6**	**P<0.0001**	**F (3, 24) = 60.38**	**P<0.0001**	**F (3, 28) = 27.32**	**P<0.0001**
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Fear > Reward, Fear +safety, Safety . Second half: Fear > Reward, Fear +safety, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Safety			
DC4	Interaction	F (3, 44) = 0.1212	P=0.9471	F (3, 24) = 0.6955	P=0.5638	F (3, 28) = 0.4701	P=0.7055
	Main effect time	F (1, 44) = 0.2963	P=0.5890	F (1, 24) = 1.4	P=0.2483	F (1, 28) = 0.3662	P=0.5499
	Main effect cue	**F (3, 44) = 117**	**P<0.0001**	**F (3, 24) = 121.8**	**P<0.0001**	F (3, 28) = 47.59	P<0.0001
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Fear > Reward, Fear +safety, Safety . Second half: Fear > Reward, Fear +safety, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Fear + Safety, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Safety	

Table 3

Comparison of Port Percentage to Each of the Cues Between the First Half and Second Half of Each DC Session for Animals That Received Systemic Administration of D1 Receptor Agonist, D1 Receptor Antagonist, or Saline

Systemic Port		Saline		Antagonist		Agonist	
DC1	Interaction	**F (3, 44) = 3.896**	**P=0.0149**	**F (3, 24) = 3.718**	**P=0.0251**	F (3, 28) = 0.6685	P=0.5785
	Main effect time	**F (1, 44) = 4.591**	**P=0.0377**	**F (1, 24) = 7.366**	**P=0.0121**	F (1, 28) = 3.836	P=0.0602
	Main effect cue	**F (3, 44) = 25.17**	**P<0.0001**	**F (3, 24) = 6.383**	**P=0.0025**	**F (3, 28) = 3.8**	**P=0.0210**
	Post hoc Bonferroni	First half > Second half: Fear		First half > Second half: Reward, Fear		N/A	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear		First half: Reward > Fear + Safety , Second half: N/A	
DC2	Interaction	F (3, 44) = 1.073	P=0.3705	**F (3, 24) = 4.957**	**P=0.0081**	**F (3, 28) = 4.441**	**P=0.0113**
	Main effect time	F (1, 44) = 0.8698	P=0.3561	**F (1, 24) = 13.14**	**P=0.0014**	F (1, 28) = 2.744	P=0.1088
	Main effect cue	**F (3, 44) = 21.1**	**P<0.0001**	**F (3, 24) = 12.2**	**P<0.0001**	**F (3, 28) = 4.525**	**P=0.0104**
	Post hoc Bonferroni	N/A		First half > Second half: Reward		N/A	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: N/A	
DC3	Interaction	F (3, 44) = 0.5187	P=0.6716	F (3, 24) = 2.532	P=0.0810	F (3, 28) = 0.3833	P=0.7658
	Main effect time	F (1, 44) = 0.2516	P=0.6185	F (1, 24) = 0.00483	P=0.9452	**F (1, 28) = 4.745**	**P=0.0380**
	Main effect cue	**F (3, 44) = 23.65**	**P<0.0001**	**F (3, 24) = 4.617**	**P=0.0109**	F (3, 28) = 1.159	P=0.3429
	Post hoc Bonferroni	N/A		N/A		No significance	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety . Second half: Reward > Fear + Safety		N/A	
DC4	Interaction	F (3, 44) = 1.35	P=0.2704	F (3, 24) = 1.096	P=0.3700	F (3, 28) = 2.409	P=0.0881
	Main effect time	F (1, 44) = 2.599	P=0.1141	F (1, 24) = 0.1642	P=0.6889	F (1, 28) = 0.001037	P=0.9745
	Main effect cue	**F (3, 44) = 33.15**	**P<0.0001**	**F (3, 24) = 9.18**	**P=0.0003**	F (3, 28) = 0.9309	P=0.4388
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		N/A	

Table 4

Comparison of Freezing Percentage to Each of the Cues Between the First Half and Second Half of Each DC Session for Animals That Received BLA Infusion of D1 Receptor Agonist, D1 Receptor Antagonist, or Saline

Cannula Freeze		Saline		Antagonist		Agonist	
DC1	Interaction	F (3, 36) = 0.6529	P=0.5864	F (3, 28) = 0.8808	P=0.4629	F (3, 32) = 0.3985	P=0.7550
	Main effect time	F (1, 36) = 0.06478	P=0.8005	F (1, 28) = 0.5322	P=0.4717	F (1, 32) = 0.05633	P=0.8139
	Main effect cue	**F (3, 36) = 7.6**	**P=0.0005**	F (3, 28) = 1.625	P=0.2059	**F (3, 32) = 4.518**	**P=0.0094**
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Fear > Reward, Safety . Second half: Fear > Reward, Fear +safety, Safety		N/A		First half: N/A . Second half: Fear > Reward	
DC2	Interaction	F (3, 36) = 0.4664	P=0.7075	F (3, 28) = 0.1847	P=0.9059	F (3, 32) = 1.025	P=0.3946
	Main effect time	F (1, 36) = 0.4238	P=0.5192	F (1, 28) = 0.07753	P=0.7827	**F (1, 32) = 5.703**	**P=0.0230**
	Main effect cue	**F (3, 36) = 8.353**	**P=0.0002**	**F (3, 28) = 11.24**	**P<0.0001**	**F (3, 32) = 5.583**	**P=0.0034**
	Post hoc Bonferroni	N/A		N/A		No significance	
	Post hoc Dunnett	First half: Fear > Reward, Safety . Second half: Fear > Reward, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Fear + Safety, Safety		First half: N/A Second half: Fear > Reward, Safety	
DC3	Interaction	**F (3, 36) = 5.809**	**P=0.0024**	**F (3, 28) = 4.34**	**P=0.0124**	F (3, 32) = 0.1307	P=0.9411
	Main effect time	F (1, 36) = 3.259	P=0.0794	F (1, 28) = 3.605	P=0.0680	F (1, 32) = 0.6263	P=0.4346
	Main effect cue	**F (3, 36) = 34.36**	**P<0.0001**	**F (3, 28) = 4.277**	**P=0.0132**	**F (3, 32) = 7.628**	**P=0.0006**
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Fear > Reward, Safety . Second half: Fear > Reward, Fear + Safety, Safety		First half: N/A . Second half: Fear > Reward, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Safety	
DC4	Interaction	**F (3, 36) = 4.094**	**P=0.0134**	F (3, 28) = 0.472	P=0.7042	F (3, 32) = 1.654	P=0.1965
	Main effect time	F (1, 36) = 4.103	P=0.0503	F (1, 28) = 3.264	P=0.0816	F (1, 32) = 0.07416	P=0.7871
	Main effect cue	**F (3, 36) = 33.79**	**P<0.0001**	**F (3, 28) = 18.78**	**P<0.0001**	**F (3, 32) = 7.072**	**P=0.0009**
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Fear > Reward, Fear + Safety, Safety . Second half: Fear > Reward, Safety		First half: Fear > Reward, Safety . Second half: Fear > Reward, Safety		First half: Fear > Reward, Safety . Second half: Fear > Safety	

Table 5

Comparison of Port Percentage to Each of the Cues Between the First Half and Second Half of Each DC Session for Animals That Received BLA Infusion of D1 Receptor Agonist, D1 Receptor Antagonist, or Saline

Cannula Port		Saline		Antagonist		Agonist	
DC1	Interaction	F (3, 36) = 0.23	P=0.8749	F (3, 28) = 1.353	P=0.2774	F (3, 32) = 1.1	P=0.3635
	Main effect time	F (1, 36) = 0.06485	P=0.8004	F (1, 28) = 1.285	P=0.2665	F (1, 32) = 1.555	P=0.2215
	Main effect cue	**F (3, 36) = 22.78**	**P<0.0001**	**F (3, 28) = 7.954**	**P=0.0005**	**F (3, 32) = 8.571**	**P=0.0003**
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety	
DC2	Interaction	F (3, 36) = 0.8141	P=0.4945	F (3, 28) = 2.187	P=0.1118	**F (3, 32) = 5.071**	**P=0.0055**
	Main effect time	F (1, 36) = 3.645	P=0.0642	F (1, 28) = 0.1068	P=0.7463	**F (1, 32) = 6.091**	**P=0.0191**
	Main effect cue	**F (3, 36) = 14.42**	**P<0.0001**	**F (3, 28) = 7.994**	**P=0.0005**	**F (3, 32) = 10.97**	**P<0.0001**
	Post hoc Bonferroni	N/A		N/A		First half > Second half: Reward	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear + Safety	
DC3	Interaction	F (3, 36) = 0.3127	P=0.8161	F (3, 28) = 0.1615	P=0.9213	F (3, 32) = 1.496	P=0.2343
	Main effect time	F (1, 36) = 2.45	P=0.1263	F (1, 28) = 3.53	P=0.0707	F (1, 32) = 0.1141	P=0.7377
	Main effect cue	**F (3, 36) = 6.996**	**P=0.0008**	**F (3, 28) = 3.729**	**P=0.0226**	F (3, 32) = 2.425	P=0.0837
	Post hoc Bonferroni	N/A		N/A		N/A	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Safety . Second half: Reward > Safety		N/A	
DC4	Interaction	F (3, 36) = 1.666	P=0.1915	F (3, 28) = 2.637	P=0.0691	F (3, 32) = 0.537	P=0.6603
	Main effect time	F (1, 36) = 0.4113	P=0.5254	**F (1, 28) = 5.039**	**P=0.0329**	F (1, 32) = 1.107	P=0.3006
	Main effect cue	**F (3, 36) = 10.92**	**P<0.0001**	**F (3, 28) = 6.369**	**P=0.0020**	**F (3, 32) = 3.687**	**P=0.0219**
	Post hoc Bonferroni	N/A		First half > Second half: Reward		N/A	
	Post hoc Dunnett	First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear, Fear + Safety, Safety		First half: Reward > Fear, Fear + Safety, Safety . Second half: Reward > Fear + Safety, Safety		No significance	

Appendix B

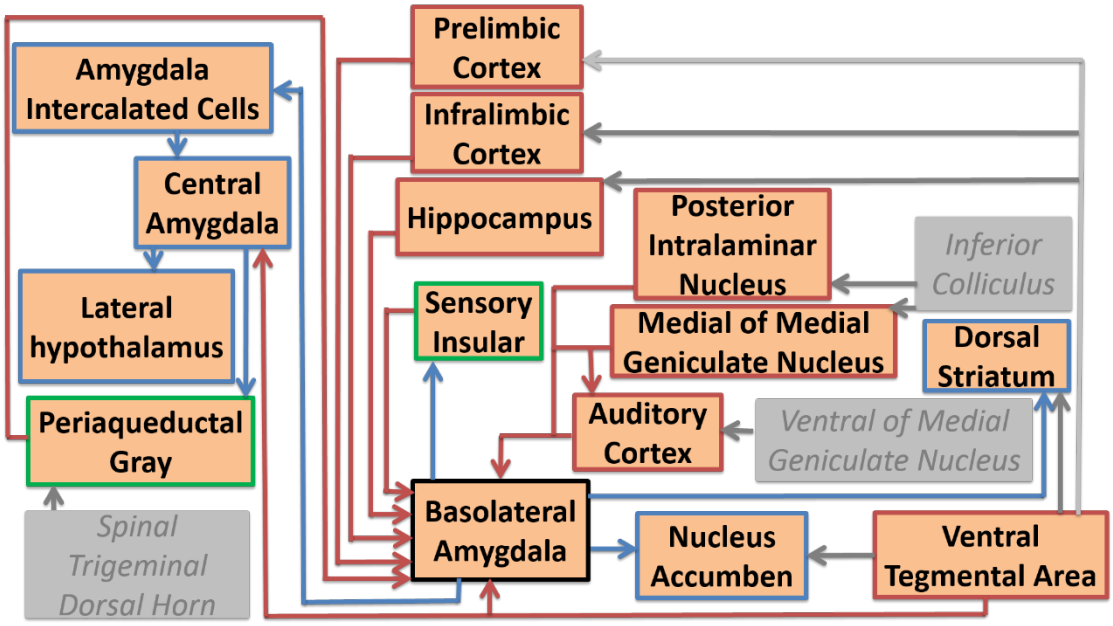
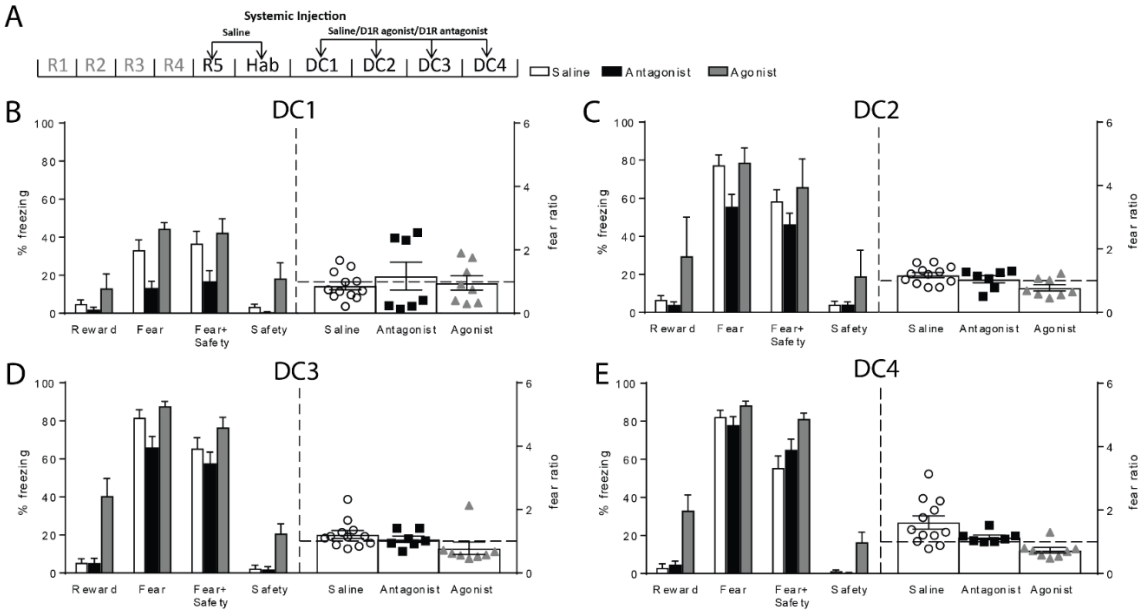
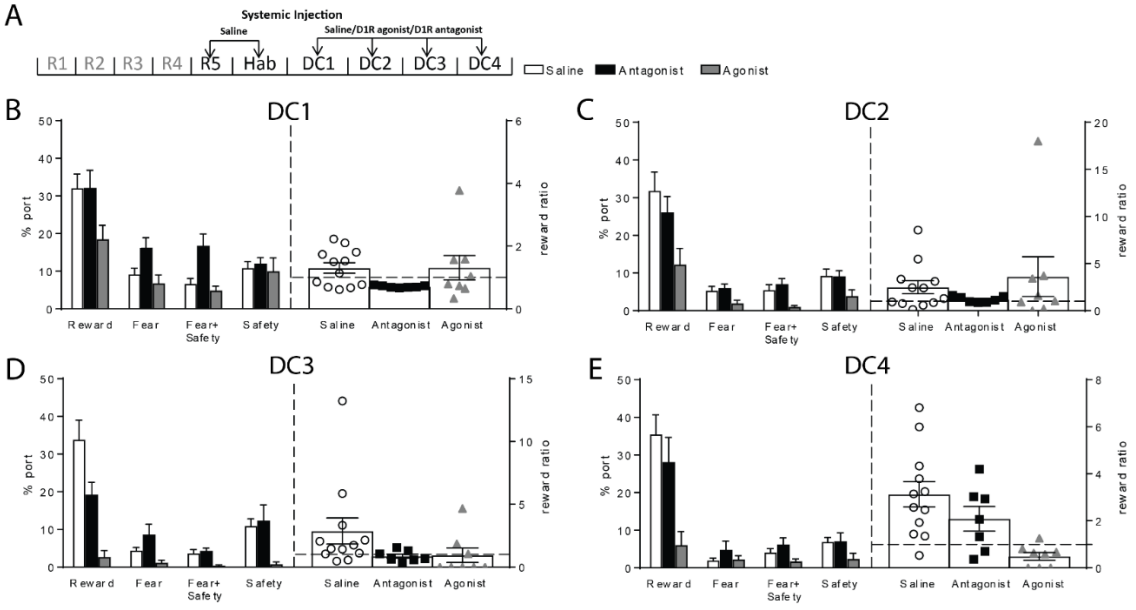


Figure 1. Wiring diagram for critical structures with upstream projections to the BLA (color red) and output projection from the BLA (color blue). Structures with reciprocal projection with the BLA are colored green.



Note. **(A)** Animals received 5 sessions of paired reward trainings followed by habituation and discrimination trainings. 20 minutes prior to the start of discrimination trainings, animals received either systemic administration of D1 receptor agonist, antagonist or saline. To habituate the animals to the injection procedure, saline injections were given 20 minutes prior to the last session of reward and habituation session. **(B-E, left panels)** Saline treated animals (n = 12) show good fear discrimination. They showed more % freezing to the fear cue than all the other cues during sessions DC2, DC3 and DC4. Systemic injections of a D1 receptor agonist (10mg/kg SKF-38393; N = 8) or antagonist (3. 33µg/kg SCH-23390; N = 7) impair fear suppression in the presence of the safety cue. Animals with systemic injections of the D1 receptor agonist or antagonist showed more % freezing to the fear cue than the reward cue and safety cues during sessions DC2, DC3 and DC4. However, neither group showed significantly more % freezing to the fear cue than the combined fear + safety cue during sessions DC1, DC2, DC3, or DC4. **(B-E, right panels)** The fear discrimination ratio was only impaired in agonist treated animals. For sessions DC2 and 4, D1 receptor agonist treated animals, but not the D1 receptor antagonist treated animals, had significantly lower fear discrimination ratios than saline animals.

Figure 2. Freezing behavior after systemic injection of dopamine D1 receptor agonist, antagonist or saline.



Note. **(A)** Training and injection paradigm is same as data presented in Figure 2. **(B-E, left panels)** Discriminative port activity to the reward cue is learned early. Animals in all treatment conditions showed more % port to the reward cue than all other cues during DC1. Systemic injection of a D1 receptor agonist (10mg/kg SKF-38393; $N = 8$), but not antagonist (3. 33µg/kg SCH-23390; $N = 7$), impairs discriminative reward seeking. Systemic injection of a D1 receptor agonist decreased % port over subsequent days of training during DC2-4. On the other hand, saline treated animals showed more % port to the reward cues than all the other cues during every DC session. The D1 receptor antagonist groups showed more % port to the reward cues than all the other cues during DC1, 2 and 4, but not DC3. **(B-E, right panels)** The reward discrimination ratio was only impaired for D1 receptor agonist treated animals not the D1 receptor antagonist during session DC4 compare to saline animals.

Figure 3. Port percentage after systemic injection of dopamine D1 receptor agonist, antagonist or saline.

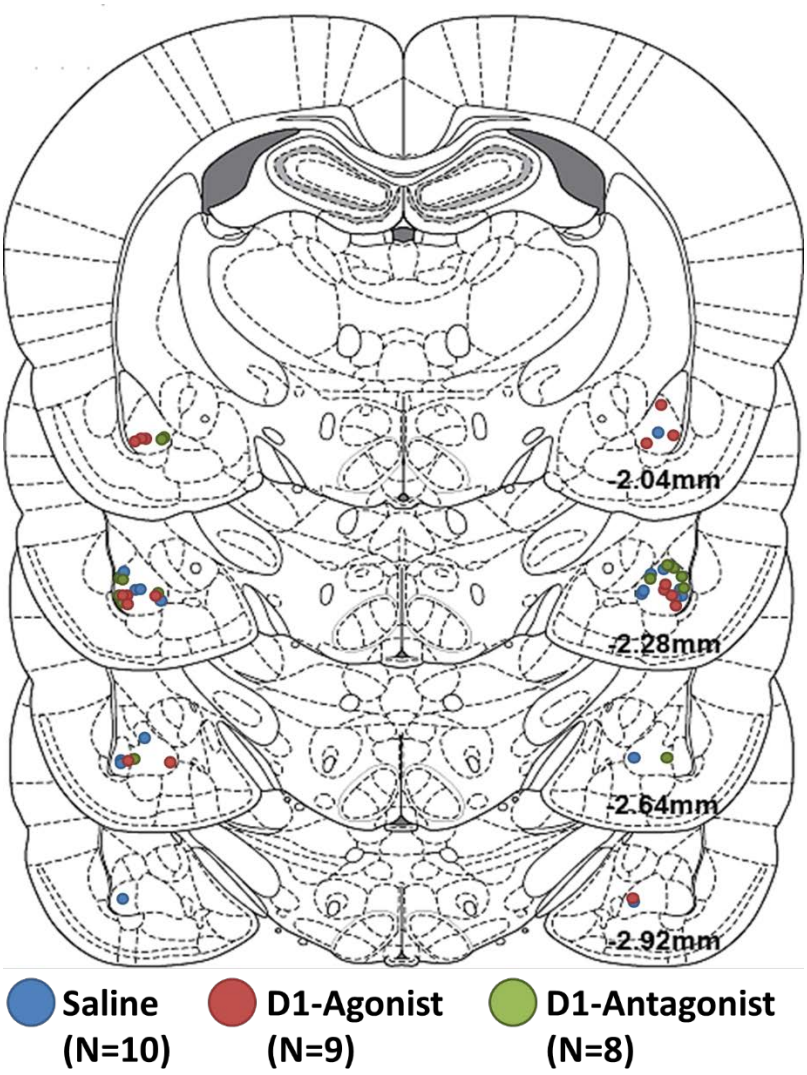
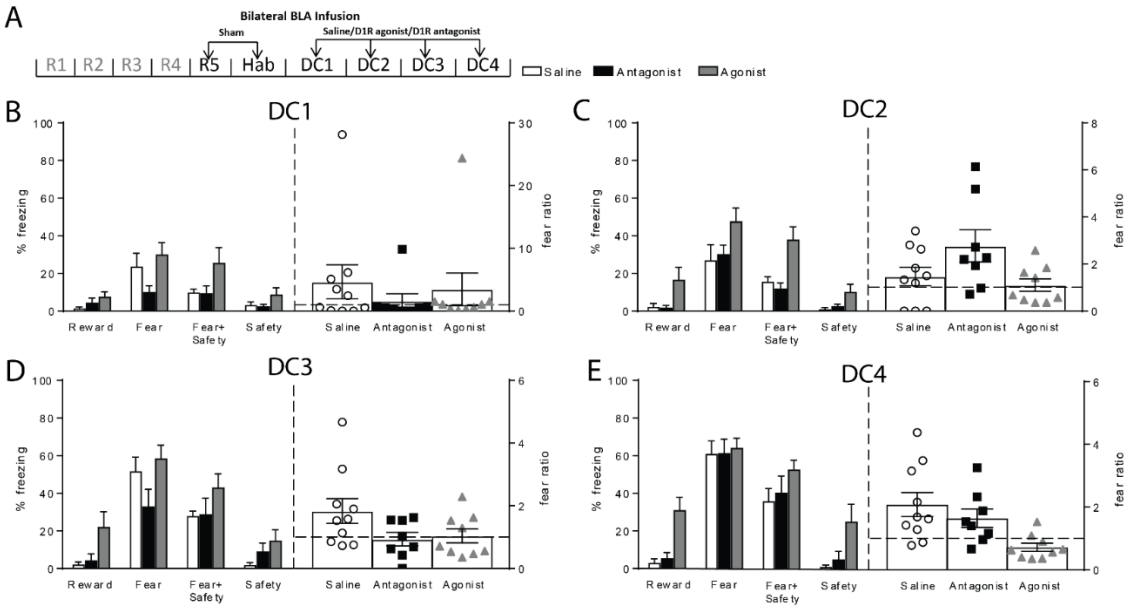
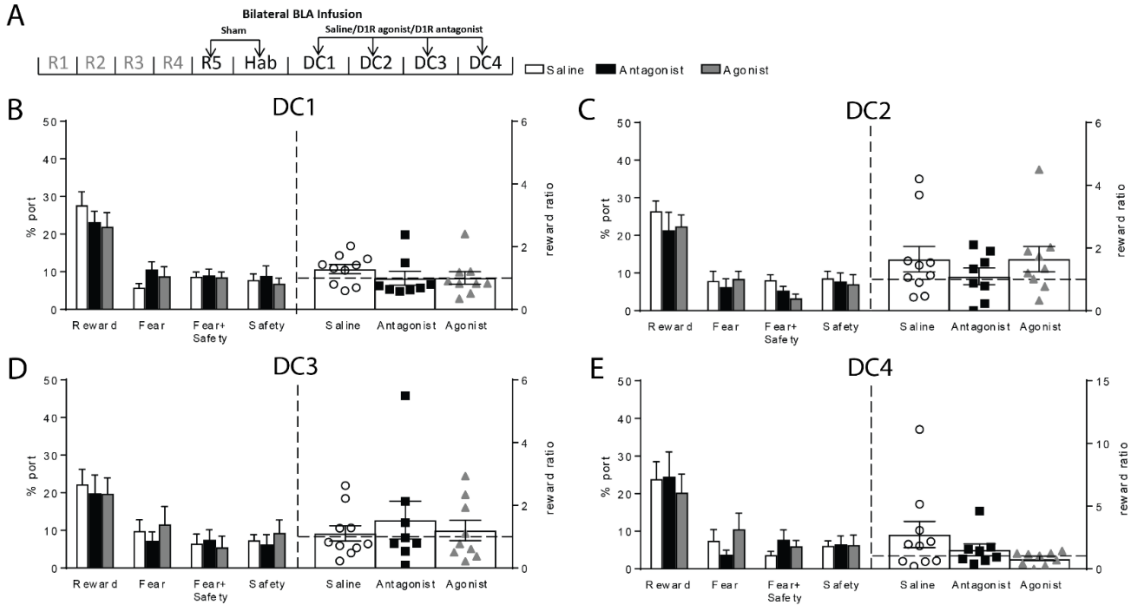


Figure 4. Placement of infusion needle tips for BLA infusion of dopamine D1 receptor agonist, antagonist or saline. Only animals with confirmed bilateral hits were included in the analysis.



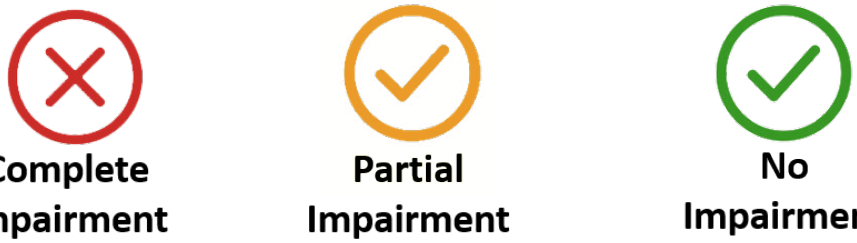
Note. **(A)** Animals received 5 sessions of paired reward trainings followed by habituation and discrimination trainings. 20 minutes prior to the start of discrimination trainings, animals received either BLA infusion of D1 receptor agonist, antagonist or saline. To habituate the animals to the infusion procedure, sham infusions were given 20 minutes prior to the last session of reward and habituation session. **(B-E, left panels)** Saline treated animals (N = 10) are showing good fear discrimination. Saline group froze significantly more to the fear cue in comparison to the other cues during sessions DC1, DC3 and DC4. Saline treated animals also showed more % freezing to the fear cue than the reward cue and safety cue during DC2. BLA infusion of a D1 receptor agonist (1 μg/0.5μL SKF-38393; N = 9), but not antagonist (0.25 μg/0.5μL SCH-23390; N = 8), impairs fear suppression in the presence of safety cue. The D1 receptor agonist group showed more % freezing to the fear cue than the reward cue and safety cue during DC1, DC2, DC3 and DC4. This group also showed more % freezing to the fear cue than the combined fear + safety cue during DC3, but not DC1, DC2 and DC4. In addition, the D1 receptor antagonist group showed more % freezing to the fear cue in comparison to the other cues during DC2 and DC4. This group also showed more % freezing to the fear cue than the reward cue and safety cue, but not the combined fear + safety cue during DC3. **(B-E, right panels)** The fear discrimination ratio was only impaired for agonist animals. During DC2 and DC4, that saline animals had higher fear discrimination ratios than agonist animals but not antagonist animals in DC4.

Figure 5. Freezing percentage after BLA infusion of dopamine D1 receptor agonist, antagonist or saline.



Note. **(A)** Training and injection paradigm is same as data presented in Figure 5. **(B-E, left panels)** Infusion of D1 receptor agonist (1 µg/0.5µL SKF-38393; $N = 9$) or antagonist (0.25 µg/0.5µL SCH-23390; $N = 8$) into the BLA does not impair discriminative reward seeking. Saline and antagonist groups exhibited more % port to the reward cue in comparison to the other cues during DC1, DC2, DC3, and DC4. The agonist group also showed more % port to the reward cue than the other cues during DC1 and DC2. This group also showed more % port to the reward cue than the fear + safety and safety cues, but not the fear cue, during DC3 and DC4. **(B-E, right panels)** BLA infusion of D1 receptor agonist or antagonist did not significantly impair reward discrimination ratio.

Figure 6. Port percentage after BLA infusion of dopamine D1 receptor agonist, antagonist or saline.



Treatment	Route	Dis Fear/Safety	Dis Fear	Dis Reward
Saline	Systemic	✓	✓	✓
	BLA	✓	✓	✓
Agonist	Systemic	✗	✓	✗
	BLA	✗	✓	✓
Antagonist	Systemic	✗	✓	✓
	BLA	✓	✓	✓

Note. Systemic D1 receptor agonist or antagonist administration impairs fear and safety discrimination. Systemic D1 receptor agonist administration also impairs fear discrimination and reward discrimination. BLA infusion of agonist but not antagonist impairs fear and safety discrimination. Reward discrimination is not affected by either BLA treatment.

Figure 7. Summary of results from systemic administration or BLA infusion of D1 receptor agonist or antagonist.