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The role of the basolateral amygdala in flexible behavior

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The role of the basolateral amygdala in flexible behavior

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DISSERTATION

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Dedication

This thesis is dedicated to everyone along the way who has helped support me throughout my scientific training, my graduate school career, and everything in between. Without the constant support of my friends, family, and co-workers, none of this would have been possible. I am eternally grateful for all of you. To Dan Linseman, you showed me how great science can be and inspired noble aspirations within me. Every day since joining your lab that fateful summer of 2003, I am thankful to have had you as a boss and friend.

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I contributed to the behavioral experiments and imaging acquisition, as well as analyzed all the behavior data, the imaging data in part, and wrote the thesis.

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The role of the basolateral amygdala in flexible behavior

Abstract of the Dissertation

Flexible behavior in response to a changing environment is required for survival, and is the physical manifestation of communication among the prefrontal cortex and different subcortical systems. The basolateral amygdala (BLA), a crucial component of the reward circuitry mediating behavioral choices, is thought to encode the value of discrete cues in the environment. In particular, the BLA is thought to be required in updating the current cue-reward association. Previous studies have shown that the BLA is involved in flexible behavior, demonstrated using reversal learning tasks, where the subject has to inhibit responding to a previously rewarded cue and start responding to a previously unrewarded cue. However, the precise role of the BLA's contribution to inhibiting responding to a previously rewarded cue or learning to respond to a previously unrewarded cue remains unclear. Furthermore, it is not known if the BLA contributes to higher order rule learning that would occur over several reversals. Therefore, we examined the role of the BLA in a 4-choice odor discrimination reversal task across several reversal phases. We found that excitoxic lesions of the BLA impaired reversal learning compared to sham-lesioned mice. BLA-lesioned mice returned more frequently to the previously rewarded odor, indicating that the BLA primarily plays a role in inhibiting response to a previously rewarded cue instead of learning a new cue-reward association. Furthermore, since the BLA acts in concert with the prefrontal cortex to produce flexible behavior, understanding the dynamics of structural connectivity from the BLA to higher-order cortical regions involved in reversal learning is the first step to elucidate how amygdalocortical

networks function. To develop our understanding of the development of these critical reward circuit connections, we used two-photon *in vivo* imaging to compare structural dynamics of axonal boutons of cortical-projecting BLA neurons in adult mice. We found that amygdala axons terminating in the dorsomedial prefrontal cortex are cortical-like in terms of their structural plasticity, and they are highly similar to orbitofrontal axons terminating in the same region. The work in this thesis elucidates a more precise role of the BLA in guiding flexible behavior, while also for the first time defining the baseline structural plasticity of axons from a limbic region projecting to a fronto-cortical region.

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Introduction

In order to survive, an animal must constantly make decisions in response to cues in the environment in order to obtain rewards and avoid aversive situations. These decisions arise from complex associations formed between cues in the environment and the outcomes they predict. These associations allow the animal to guide their behavior in response to an ever-changing set of factors, both internal and external. For example, a sated animal will no longer seek food, while a hungry animal may forego its meal in the presence of a predator.

The ability to act flexibly in response to changes in the environment requires a network of regions in the brain to learn associations between cues and their respective outcomes as well as to exchange information with other areas. Two of these areas, the basolateral amygdala (BLA) and the orbitofrontal cortex (OFC) are known to guide flexible behavior – the ability to change behaviors in response to changes in the environment (Churchwell et al., 2009; Schoenbaum et al., 2003; Ragozzino, 2007). However, the precise role of the BLA in guiding flexible behavior remains unclear. By expanding on a previously used behavioral paradigm, we can more clearly elucidate the precise role of the BLA in guiding flexible behavior.

The basolateral amygdala

The amygdala is a part of the limbic system and is comprised of 13 nuclei, which when grouped together based on cytoarchitecture and fiber connections, make up three divisions of the amygdala: the centromedial amygdala, the basolateral amygdala (BLA), and the cortical

amygdala (Swanson and Petrovich, 1998; Knapska et al., 2007). Interestingly, while the three regions are distinct from one another, they each are highly similar to nearby brain regions. The centromedial amygdala resembles the ventral striatum, the cortical amygdala resembles the caudal olfactory cortex, and the basolateral amygdala is thought to be an extension of the deep cortex (Knapska et al., 2007; Ledoux, 2004). The BLA is comprised of the lateral, basal, and accessory basal nuclei and highly resembles the cortex in terms of its cellular composition, afferent synaptic targets, and efferent projection targets (Carlsen and Heimer, 1988; Ledoux, 2004). Immunohistochemical and electrophysiological analyses have identified the predominant cell type of the BLA as excitatory pyramidal projection neurons, with a small proportion of neurons being local inhibitory neurons (McDonald, 1982, 1984; Washburn et al., 1992).

Through its afferent and efferent connections throughout the brain, the amygdala supports processing of sensory, visceral, and emotional information, all of which help guide behavior (Knapska et al., 2007; Ledoux, 2004). The basolateral complex receives inputs from cortical, hippocampal, thalamic, and striatal regions among others, and in turn provides output to cortical, hippocampal, ventral striatum, and hypothalamic regions and the central amygdala (Knapska et al., 2007). These connections underlie the capacity of the BLA to act both as an association center, integrating information from sensory modalities about predictive cues and their respective outcomes, and an emotional processing center, attaching emotional significance to experiences and motivating future responses (Benes, 2010).

The role of the BLA in behavior

Humans with bilateral lesions of the BLA, due to the rare genetic disease Urbach-Weith or to brain damage from other causes, demonstrate impaired facial expression recognition (de Gelder et al., 2014), altered emotional processing (Tranel et al., 2006; Hurlemann et al., 2007), increased risk-taking (Talmi et al., 2010), and poor decision-making skills (Bechara et al., 1999; Brand et al., 2007). Furthermore, the amygdala is involved in mediating arousal in response to emotional events. Humans with amygdala damage fail to develop skin conductance responses (SCRs) in response to aversive stimuli or risky situations, possibly contributing to poor decision-making skills and higher levels of risk-taking (Bechara et al., 1999). In rodents, neuronal activity in the BLA (as well as the central nucleus) correlated with increases in blood pressure, a measure of arousal, after a learned appetitive or aversive cue presentation (Shabel and Janak, 2009). These and other studies indicate that the BLA influences the emotional aspect of learning, thus making an experience more likely to be remembered.

In this context, it is not surprising that the BLA was primarily known for its role in fear conditioning, a process of emotional learning whereby a neutral stimulus evokes a fear response after being paired with an aversive stimulus. Through lesion, pharmacological, and electrophysiological experiments, the BLA has been shown to be involved in the learning of the association between the neutral stimulus and the aversive stimulus, the long-term storage of the association, and the extinction of the behavior (LeDoux, 2003; Phelps and LeDoux, 2005; Fanselow and Ledoux, 1999; Barad et al., 2006).

Only recently have studies focused on the role of the BLA in reward learning. Early work using aspiration lesions indicated that the BLA was necessary for stimulus-reinforcement

associations (Jones and Mishkin, 1972). However, more selective lesions, together with electrophysiological, pharmacological, and optogenetic studies analyzing the role of the BLA in a variety of paradigms, have uncovered that the role of the BLA in reinforcement behavior is in fact multi-purpose and more complex than solely acting as an association center for stimuli and their outcomes (Baxter and Murray, 2002). For example, the BLA alters decision-making (Ghods-Sharifi et al., 2009), promotes reward-seeking behavior (Ambroggi et al., 2008; Stuber et al., 2011), drives goal-directed behavior (Johnson et al., 2009; Hatfield et al., 1996), and supports second-order conditioning (Hatfield et al., 1996).

Neurons in the BLA encode multiple properties of cues and outcomes

In order to make an association between a cue and an outcome, multiple properties of the cue and outcome need to be encoded within the brain. Neurons in the primate or rodent amygdala fire in response to sensory properties of stimuli, particularly when the stimulus is novel (Baxter and Murray, 2002; Nishijo et al., 1988; Uwano et al., 1995). Neurons that respond to multiple modalities cluster in the BLA and central nucleus (Uwano et al., 1995). Several studies have reported that the amygdala encodes the intensity, value, and valence of the stimuli or the predictive cues (Paton et al., 2006; Small et al., 2003; Anderson et al., 2003; Salzman et al., 2007; Parsana et al., 2012; Kuemerle et al., 2007; Young and Williams, 2013), as well as the unexpected presentation or omission of the reward itself (Roesch et al., 2010; Tye et al., 2010). BLA neurons also encode motivational and incentive properties of cues (Tye and Janak, 2007b). The fact that all these properties of cues and outcomes are encoded within the BLA strongly indicates that learned associations of cues and outcomes happen locally.

The BLA and flexible behavior

Though the BLA is not necessary for simply learning an association between a cue and a reward (Machado and Bachevalier, 2007; Schoenbaum et al., 2003; Churchwell et al., 2009), two behavioral paradigms demonstrate that the BLA is needed to maintain a representation of the *current* cue-reward association in order to guide behavior. The first example of this is reinforcer devaluation.

In reinforcer devaluation paradigms, an animal learns to respond (through Pavlovian or instrumental training) to two cues, both of which predict an equally satisfying but distinct reward. After learning, the animal is returned to its homecage where one of the rewards is devalued, either by allowing the animal to consume as much as they want of it (satiation), or by pairing the reward with lithium chloride (LiCl) to make them sick. When returned to the test chamber and presented with each of the two cues in the absence of reward, control animals decrease responding to the devalued cue while maintaining responding to the other cue. In contrast, animals with the BLA lesioned or inactivated do not show this decrease in responding to the devalued cue (Johnson et al., 2009; Hatfield et al., 1996; West et al., 2012). Importantly, after the test, both groups of animals are given *ad libitum* access to the rewards (no cues) and here, BLA-lesioned animals will largely ignore the devalued reward, just like controls. In other words, animals with BLA lesions no longer act in a goal-directed manner, since they still respond to the cue even though when given the opportunity, they do not consume the devalued reward. Furthermore, BLA lesions after the devaluation of the reward but before the devaluation test do not cause impairment in the devaluation test. These studies demonstrate

that not only does the presentation of the cue evoke a representation of the rewarded outcome, but that the BLA is necessary to update the cue-reward association. The animal, having knowledge of the devalued reward (either by satiation or LiCl-induced illness) also updates and devalues the cue. However, BLA lesions impair the inability to update the cue-reward association, thus preventing the animal from acting flexibly by changing its behavior to a changed contingency in its environment.

Another way to assess the role of the BLA in flexible behavior by requiring the animal to update cue-reward associations is through a task called reversal learning. In reversal learning, an animal learns that one cue predicts reward while another does not. After learning this contingency, the contingencies are switched so that the rewarded cue is no longer rewarded and the unrewarded cue is now rewarded. The animal has to learn to inhibit responding to the previously rewarded cue and start responding to the previously unrewarded cue. Lesion and inhibition studies of the BLA on 2-choice reversal learning tasks have demonstrated that the BLA is critically involved in reversal learning (Izquierdo et al., 2013; Schoenbaum et al., 2003; Churchwell et al., 2009). However, two of these studies arrived at different conclusions regarding the nature of the impairment, with one study concluding that inhibition of the response to the previously rewarded cue was impaired (Churchwell et al., 2009), while the other study concluded that the learning of the new cue-reward association was impaired (Schoenbaum et al., 2003). Since appropriate behavioral actions in the presence of multiple stimuli requires response inhibition to unrewarded cues and exploratory behavior to seek out newly rewarding cues, gaining a better understanding of the role of the BLA in either of these two actions is useful to understand how the BLA guides flexible behavior. To address this

conflict, we used an expanded reversal learning paradigm where the animal has to make a decision among four cues to receive a reward. In the first chapter of this thesis, we attempt to clarify the role of the BLA in flexible behavior by modifying an existing reversal learning paradigm to dissociate inhibition to the previously rewarded cue from learning to respond to the previously unrewarded cue. Further, we ask what the role of the BLA is when the animal undergoes multiple reversals across several days. By testing the animal's ability to reverse contingencies multiple times over several days, we can examine if the BLA is involved in guiding an animal's behavior only when learning a new set of cue-reward predictions or if the BLA is implicated in learning a higher order "rule of reversal."

The orbitofrontal and dorsomedial prefrontal cortex

As mentioned above, the BLA does not work in isolation to guide flexible behavior. In humans and in animals, several behaviors modulated by the BLA are also regulated by the OFC (Schoenbaum et al., 2003; Bechara et al., 1999; Baxter et al., 2000; West et al., 2011) and the dorsomedial prefrontal cortex (dmPFC) (Ragozzino and Rozman, 2007; Floresco and Ghods-Sharifi, 2007; Ishikawa et al., 2008). Furthermore, flexible behavior requires encoding of both an action-outcome relationship and a cue-outcome relationship. The dmPFC is thought to encode action-outcome (Rushworth et al., 2004, 2007), and inactivation studies using effort-based tasks indicate that a BLA-dmPFC pathway is responsible for weighing the value of an expected outcome compared to the value of the action (Floresco and Ghods-Sharifi, 2007). The OFC encodes both a representation of the cue-outcome as well as the reward expectation (Rushworth et al., 2007). Finally, the BLA has strong reciprocal connections with the OFC and

dmPFC (Krettek and Price, 1977; Carmichael and Price, 1995a; Öngür and Price, 2000). Thus, understanding how these areas communicate with one another will provide insight into how these areas work together to guide behavior. One manner by which we can analyze this is through 2-photon *in vivo* imaging, which allows for the repeated imaging of axons, axonal boutons, dendrites, or dendritic spines over long periods of time to reveal changes in synaptic connections.

Structural plasticity of axons

In addition to understanding the role of the BLA in guiding flexible behavior, we aimed to understand the structural plasticity of axons projecting from the BLA to the dmPFC, a cortical region involved in flexible behavior (Ragozzino and Rozman, 2007; Johnson and Wilbrecht, 2011). The development of 2-photon laser scanning microscopy to image neuronal projections over long periods *in vivo* has greatly increased our understanding of structural plasticity, at baseline, in experience-dependent tasks, and with disease (Kim et al., 2012; Sigler and Murphy, 2010; Knott and Holtmaat, 2008; Pan and Gan, 2008; Bhatt et al., 2009; Holtmaat and Svoboda, 2009; Svoboda and Yasuda, 2006). By imaging axons or dendrites repeatedly, we can analyze the density, and rate of formation and elimination of dendritic spines and axonal boutons, the apposition of which form a synapse. Studies have demonstrated that there is a low level of continual plasticity in multiple brain regions, even well into adulthood (Zuo et al., 2005; Holtmaat et al., 2005; Trachtenberg et al., 2002; Grutzendler et al., 2002; Stettler et al., 2006; De Paola et al., 2006; Knott et al., 2006). Furthermore, a number of these studies have also included ultra-structural analysis on the imaged spines or boutons to demonstrate that the

spines or boutons do, in fact, form synapses (Trachtenberg et al., 2002; Knott et al., 2006; De Paola et al., 2006). Studies analyzing spine or bouton dynamics over time provide new insight to the plasticity of the brain under normal conditions, in a learning context, and in disease.

Information regarding structural plasticity in either the BLA or OFC is critical for our understanding of how experiences modify connections within these regions. The first step towards understanding these changes in plasticity requires knowledge of baseline structural characteristics of the axons in either the BLA or OFC. In chapter two of this thesis, we compare structural plasticity of both of these axons in the dmPFC, the third region implicated in the particular behavioral task we used in the first chapter of this thesis.

In the first chapter of this thesis, we focus on the role of the basolateral amygdala in flexible behavior. By expanding the typical 2-choice reversal paradigm to a set of 4 choices, we can dissociate the role of the BLA in inhibiting responding to a previously rewarded cue from learning to respond to a previously unrewarded cue. We show that the BLA is primarily involved in inhibiting responding to the previously unrewarded cue. Furthermore, when the animals are challenged to multiple reversals over several days, we found that the BLA is only necessary for reversal learning in the earlier reversal phases, coinciding with the learning of new cue-reward associations and potentially delaying higher order rule learning.

In the second chapter of this thesis, we use two-photon *in* vivo imaging to examine the baseline structural plasticity of BLA axons to the dmPFC and compare them with axons

originating from the OFC. We found that BLA axons are cortical-like in their structure and bouton dynamics, with comparable turnover rates to axons originating from the OFC.

In the final chapter of this thesis, we place our results in the context of the broader literature, with the aim to explain how the BLA, OFC, and dmPFC work together to guide flexible behavior. We then discuss future experiments that will further our understanding of these three regions' interactions. The work in this thesis elucidates a more precise role of the BLA in guiding flexible behavior, while also for the first time defining the baseline structural plasticity of axons *in vivo* of a limbic region.

Chapter One

Basolateral amygdala lesions disrupt serial reversal learning by impairing inhibiting responding to a previously rewarded cue

Abstract

Flexible behavioral actions in response to a changing environment are required for survival. The basolateral amygdala (BLA), a crucial component of reward circuitry mediating behavioral choices, is thought to represent the value of cues in the environment. In particular, the BLA is thought to act as a "pay attention" signal to other areas of the brain, with neuronal activity in the BLA representing a change in expected outcome versus actual outcome of reward after a reward-predictive cue is presented. Previous studies have demonstrated that the BLA is involved in reversal learning tasks, a measure of flexible behavior where the subject has to inhibit responding to a previously rewarded cue and start responding to a previously unrewarded cue. However, the precise role of the BLA's contribution either to inhibiting responding to a previously rewarded cue or learning the new cue-reward association remains unclear. Additionally, it is unknown if the BLA contributes to the higher order rule learning that would occur over several reversals. By testing BLA-lesioned animals on multiple reversals over several days, we can begin to address if the BLA is involved in higher order learning (e.g., learning the rule that when a previously rewarded cue is no longer rewarded, switch cues immediately) versus if it's involved solely when learning a novel cue-reward association. Therefore, we examined the role of the BLA in a 4-choice odor discrimination reversal task (4-choice ODT) across several reversal phases. We found that excitotoxic lesions of the BLA

impaired reversal learning compared to sham-lesioned mice. BLA-lesioned mice returned more frequently to the previously rewarded odor, but did not show impaired learning of the new cue-reward association. These data indicate that the BLA primarily plays a critical role in devaluing a previously rewarded cue, while learning a new cue-reward association remains intact.

Introduction

Flexible behavior in response to constantly changing cues in the environment is critical for an animal's survival. This flexible behavior is evident in reversal learning paradigms, where animals have to stop responding to a previously rewarded cue, and start responding to a previously unrewarded cue (Fig 1A). Lesion and inhibition studies on 2-choice reversal learning tasks have demonstrated that the BLA is critically involved in reversal learning (Izquierdo et al., 2013; Schoenbaum et al., 2003; Churchwell et al., 2009). In olfactory discrimination tasks, lesions or inhibition of the BLA impaired reversal learning (Schoenbaum et al., 2003; Churchwell et al., 2009). However, these studies arrived at different conclusions regarding the nature of the impairment, with one study concluding that inhibition of the response to the previously rewarded cue was impaired, while the other study concluded that the learning of the new cue-reward contingency was impaired (Fig 1B). Importantly, these conclusions underscore the presence of three necessary behavioral actions necessary for flexible behavior: 1) response inhibition, suppressing an action that is no longer necessary 2) exploratory behavior, seeking out alternative cues in the environment that predict reward, and 3) maintenance, choosing to return to the newly rewarded cue repeatedly.

One possibility for the conflicting conclusions is the nature of the task itself. In one study, a 2-choice task was used where both cues were presented simultaneously (Churchwell et al., 2009). With this set-up, if the animal makes an error, the error is necessarily to the previously rewarded cue. Thus, it is difficult to differentiate if the impaired reversal learning is due to an inability to inhibit the response to the previously rewarded cue or an inability to learn the new cue-reward association (and thus opting to still make a response, albeit to the previously rewarded cue). In contrast, in another study, a go/no-go paradigm was used, where one cue was presented at a time, allowing the animal to make a decision about each individual cue without interference from the second cue (Schoenbaum et al., 2003). In this situation, one can distinguish between impairments in inhibiting a response to a previously rewarded cue versus impairments in learning to respond to a previously unrewarded cue since the animal is being asked to evaluate only one cue per trial. However, individual presentation of the cues does not subject the animal to the same competing nature of choosing between cues as might be found in real life.

A solution to address these caveats is to present the animal with more than two cues simultaneously with only one cue being rewarded. With this set-up, the animal is given the option to make an error that is not necessarily tied to a previously learned association. If a BLA-lesioned animal inhibits their response to a previously rewarded cue but continues to make errors to other unrewarded cues, it suggests that the animal can in fact inhibit responding to a previously rewarded cue but may be impaired learning the new cue-reward association. Alternatively, if even when alternate cues are presented, the animal continues to respond to

the previously rewarded cue, this suggests that the animal is impaired at inhibiting responding to the previously rewarded cue or at initiating exploratory behavior.

Another question that can be addressed using reversal learning paradigms is determining the role of the BLA in learning the higher order "rule of reversal," whereby an animal learns that once a cue is no longer rewarded, the best strategy is to switch to the alternative cue. This can be seen in serial reversal paradigms, where the cue-reward associations are repeatedly switched (Fig 1A). Interestingly, despite finding that lesions of the BLA impaired performance in the first reversal phase, lesions of the BLA have not been shown to impair serial

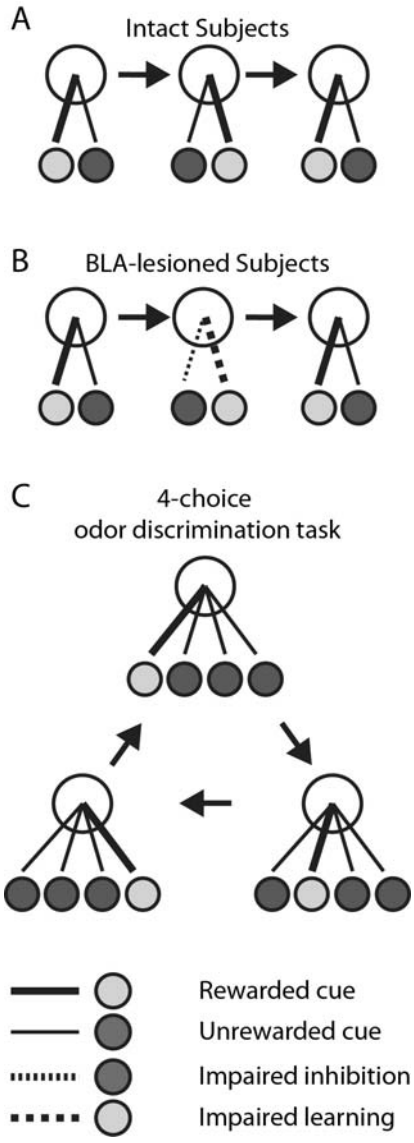

Figure 1. Schematics of 2-choice versus 4-choice odor discrimination tasks (ODT). (A) In a 2-choice ODT, subjects must first learn that one cue is rewarded (heavy bar, light gray circle) and the 2nd cue is not (light bar, dark gray circle). Large white circles represent the context of the behavioral arena. During the reversal phase, the contingencies are switched. In serial reversal learning, the subject is switched back to the original contingencies. **(B)** BLA lesion or inactivation may impair either the inhibition to the previously rewarded odor or learning of the new cue-reward association. BLA lesions did not affect the 2nd reversal. **(C)** A 4-choice ODT is used to address the conflicting conclusions of the role of the BLA in reversal learning. First, the presentation of 4 cues more closely mimics a real world environment where a subject might encounter multiple cues simultaneously. Second, the subject is not restricted to responding solely to the previously rewarded odor. Finally, the subject is presented with two new cue-reward contingencies and then returned to previously learned cue-reward contingencies.

reversal learning (Schoenbaum et al., 2003), (Fig 1B). These data indicate that the BLA was involved in updating and learning a new cue-reward association, but not in learning the "rule of reversal" per se. An alternative conclusion, however, is that in this 2-choice serial reversal learning paradigm, the animal returns to a previously learned set of cue-reward predictions. Thus, other brain regions, such as the OFC, could be involved in guiding the behavioral outcome when the animal is returned to a previously learned contingency, thereby compensating for the lesioned BLA (Wilson et al., 2014). One way to resolve this issue would be to compare the effects of BLA lesions on reversal learning when the animal is repeatedly switched to new cue-reward contingencies versus returning to previously learned cue-reward contingencies.

We addressed these two questions by using a 4-choice odor discrimination task (4-choice ODT) (Ragozzino and Rozman, 2007; Kim and Ragozzino, 2005; Johnson and Wilbrecht, 2011) (Fig 1C). In this task, the animal is simultaneously presented with four pots of distinctly-scented bedding, with only one odor predicting the presence of a Cheerio reward, buried underneath the bedding. With four odors presented simultaneously as opposed to two, the animal no longer has to make an error to the previously rewarded cue in the reversal phase, but can instead choose to dig in any of the three unrewarded pots. This expansion of choice will allow us to determine if the BLA is necessary for inhibiting responding to the previously rewarded cue (i.e., continually returning to the previously rewarded cue despite no reward), for learning the new cue-reward association (i.e., initiate exploratory behavior on a similar timescale as controls, but not maintain responding to the newly rewarded cue), or for causing a general impairment in learning to all of the cues (i.e., respond to all cues equally regardless of reward outcome). In addition to dissociating the response pattern, a 4-choice ODT may task the

brain in a different manner than a 2-choice task; since a 4-choice task requires a greater cognitive load, it may be a more sensitive task or tax different circuits. Indeed, previous studies analyzing the effects of dmPFC inhibition in reversal learning found that dmPFC inactivation only has an impact on a 4-choice ODT but not a 2-choice ODT (Ragozzino and Rozman, 2007). Furthermore, OFC inhibition led to a different set of errors between the 2-choice and 4-choice ODT (Kim and Ragozzino, 2005). Thus, analyzing the effect of BLA lesions in a 4-choice ODT may provide additional information regarding the role of the BLA in a more complex scenario.

Finally, we tested BLA-lesioned mice on a series of reversals so that two new cue-reward associations were learned followed by returning to previously learned cue-reward associations. This allows us to distinguish the role of the BLA in circumstances where a new association is being learned versus returning to previously-learned contingency patterns.

We hypothesized that the BLA is necessary for successful flexible learning in a serial reversal task if the animal had to learn a new cue-reward contingency. Furthermore, we hypothesized that the impairments seen in reversal learning are primarily due to inhibiting a response to a previously rewarded cue and not due to impairments of learning the new cue-reward association. Finally, we predict that if returned to a previously learned cue-reward contingency, the BLA will no longer be necessary for successful behavior. We found that BLA lesions impair serial reversal learning, specifically in phases where a new cue-reward contingency was introduced. This impairment was due to an inability to inhibit responding to the previously rewarded cue, while learning the new cue-reward association was unimpaired. Furthermore, once the animal was returned to a cue-reward contingency they had previously learned, BLA lesions did not cause overt impairments in behavior.

Methods

2.1 Animals

Eight-week old male C57BL/6 mice were ordered from Jackson Laboratories and were housed on a 12:12h reverse light-dark cycle with lights on at 10 P.M. Mice were housed five per cage with nesting material. Food restriction began two days before behavioral pre-training and at least 3 days after surgery. During food restriction, mice were given 1.0–1.9 g of food per day to reach 85% body weight and ranged from 18.6–26.2 g on the first day of reversal. Water was freely available both in the homecage and in the maze during all phases of behavioral testing. All animal procedures were approved by the University of California, Berkeley Institutional Animal Care and Use Committee.

2.2 Basolateral amygdala lesions

Bilateral stereotactic lesions were made in the basolateral amygdala of adult mice at least three days before food restriction. Lesions were made under isoflurane anesthesia using established coordinates (Franklin and Paxinos, 2008). Using a Nanoject II injector (Drummond Scientific Company, Broomall, PA), 0.1 µl of NMDA (20 mg/ml in sterile 0.9% saline) was injected bilaterally (AP: -1.3mm; ML: ±3.35mm; V: 4.25mm). For sham surgeries, saline vehicle was injected at the same coordinates. Prior to surgery and during recovery, mice were given access to 0.5 mg/ml cherry-flavored acetaminophen solution (Perrigo, Allegan, MI) and 0.7 mg/ml oral sulfamethoxazole with 0.1 mg/ml trimethoprim antibiotic solution (Hi-Tech Pharmacal, Amityville, NY) in drinking water.

2.3 Apparatus

Animals were trained on a 4-choice maze (described in Johnson and Wilbrecht, 2011). Briefly, each quadrant of a 12”×12”×9” acrylic chamber contained a white ceramic pot filled with distinctly-scented bedding on testing days (Fig 3B). The pots were sham baited with a Honey Nut Cheerio (General Mills, Minneapolis, MN) secured underneath a mesh screen at the bottom. Between trials, a 6” diameter removable cylinder fit in the center of the maze isolated the mouse from the rest of the maze.

2.4.1 Pre-training procedure

The 4-choice odor discrimination and reversal task was used as previously described (Kim and Ragozzino, 2005; Johnson and Wilbrecht, 2011). On the first day of pre-training, mice were habituated to the chamber and ceramic pots (without bedding), each containing a small piece of Honey Nut Cheerio (approximately 10 mg each). The mouse was placed in the start cylinder in the center of the chamber. Once the cylinder was lifted, the mouse was allowed to explore the maze and to eat the cheerio reward in the pots. After 10 minutes, the mouse was returned to the start cylinder and the pots were re-baited. This procedure was repeated three times for a total habituation time of 30 minutes. The maze was wiped with 70% ethanol between animals.

The second day of pre-training was a shaping session to teach the mice to dig to find cereal pieces buried in coarse pine wood shavings (Kaytee Products, Inc., Chilton, WI). One pot with increasing amounts of wood shavings covering the cereal reward was used in this shaping phase. The quadrant containing the pot was alternated in each trial (SE to NW to SW to NE) and

all quadrants were rewarded equally. Trials were untimed and consisted of two trials with no shavings covering the cereal piece followed by two trials with a dusting of shavings, and four trials each where the pot was a quarter full, half full, and finally with the cereal piece completely buried.

2.4.2 Four-choice odor discrimination acquisition

Wood shavings were scented on the day of testing. Anise extract (McCormick, Hunt Valley, MD) was used undiluted at 0.02 ml/g of shavings. Clove, litsea, and eucalyptus oils (San Francisco Massage Supply Co., San Francisco, CA) were diluted 1:10 in mineral oil and mixed at 0.02 ml/g of shavings. Thymol ("thyme"; Alfa Aesar) was diluted 1:20 in 50% ethanol and mixed at 0.01 ml/g of shavings. The rewarded odors used in the initial discrimination and reversal phases are listed in Table 1 (01=Odor 1, etc.).

The day after the shaping pre-training, the mouse was trained on the four-choice odor discrimination "acquisition" phase as previously described (Johnson and Wilbrecht, 2011). Briefly, mice had to discriminate amongst four different odors, only one of which predicted the presence of a buried cheerio reward. Pot location was changed pseudo-randomly on every trial so no spatial strategy could be used to learn the task. At the beginning of every trial, the start cylinder was removed and the mouse was allowed to explore the chamber and each of the four pots. A trial ended when the mouse dug in a pot. On a correct trial, the mouse was allowed to eat the cheerio before being returned to the start cylinder. On an incorrect trial the mouse was immediately returned to the start cylinder. Digging was defined as purposefully moving the shavings with both front paws, but not as superficial sniffing or chewing of the shavings. Entries

were counted if two or more paws were in the quadrant. Pots were re-baited between trials if necessary. If no digging choice was made after five minutes, the trial was terminated, the mouse was returned to the start cylinder, and an omission was recorded. Analysis of entries based on trial outcome did not include omissions. When the mouse reached criterion, defined as completing 8 out of 10 consecutive trials correct (including omissions), the animal was briefly placed in a container containing scented shavings from each of the different odors to prevent transmission of an odor preference to its cagemates before being returned to its homecage.

2.4.3 Recall

Twenty-four hours after acquisition, the mouse was tested on the same odor-reward contingency as the previous day, with the previous day's odor being rewarded. Once the mouse reached criterion, the reversal phase began immediately.

Table 1. Cheerio-baited odors for acquisition, recall, and reversal phases.

	Acquisition	R1	R2	R3	R4	R5
Recall Odor	-	O1	O2	O5	O1	O2
Rewarded Odor	O1	O2	O5	O1	O2	O5

2.4.4 Serial reversal procedure

Mice went through five different days of reversal phases (R1-R5) (Fig 2A). During the serial reversal phase, mice went through recall of the previous day's odor-reward contingency before

immediately entering the new reversal phase. The rewarded odors used in each phase are listed in Table 1. All shavings were replaced with new shavings in new pre-prepared pots (separate from the recall phase) to prevent discrimination from unintentional cues. Switching in the new pots between recall and reversal phases took less than one minute, approximately the same amount of time as moving the pots on a regular trial, thus being unlikely to give the animal an unintentional cue that the odor-reward contingency was being switched. On the first reversal phase (R1), a novel odor was swapped in for the 4th odor presented during the acquisition phase (Fig 2A). On subsequent reversal phases (R2-R5), all odors remained the same. Novel odors were not introduced in subsequent phases so as not to act as an unintentional cue to the animal that the contingencies were being switched. The odors and associated errors are listed in Table 2. Once the animal reached criterion of the reversal phase (8 out of 10 consecutive trials correct), the animal was briefly placed in a container containing scented shavings from each of the different odors then returned to its homecage.

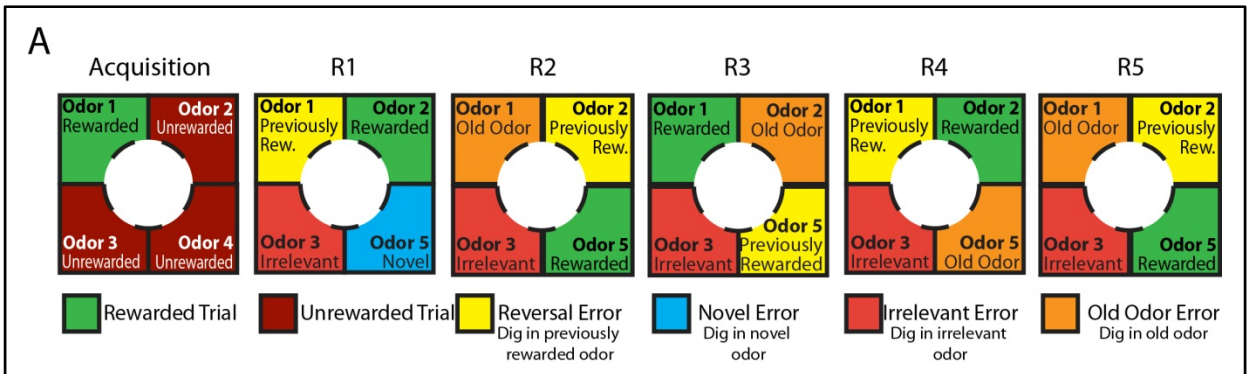

Figure 2. Schematic of each behavioral phase and the corresponding error types. Acquisition: Green is rewarded, Brown is unrewarded. Reversal phases: Green is rewarded. Yellow is previously rewarded, resulting in a reversal error. Blue is a novel odor (only in R1), resulting in a novel error. Red is a continually unrewarded odor, resulting in an irrelevant error. Orange (R2-R5) is the odor that was rewarded two phases prior, resulting in an old odor error.

2.5 Histology

All mice were transcardially perfused with 4% paraformaldehyde in PB (0.1M, pH 7.4) within a week after the last reversal phase was completed. Brains were removed and placed in 4% paraformaldehyde overnight. Brains were then transferred to a 30% sucrose solution for 48 hours. Tissue was frozen on dry ice before coronal sections (40 µM thick) were cut on a microtome. Sections were mounted and allowed to dry for at least 48 hours before stained with Nissl (Fig 3A). Lesioned animals were included in the behavioral analysis if at least 30% of the BLA was lesioned over 120 µM.

2.6 Statistical analysis

Values are reported as mean (*M*) ± SEM. For the acquisition phase, two-tailed t-tests were used for comparison. For comparison during recall or across reversals, two-way repeated-measures

Table 2. Error type by odor for each reversal phase.

	Error Description	**R1**	**R2**	**R3**	**R4**	**R5**
Reversal	Dig in the previously rewarded odor	O1	O2	O5	O1	O2
Perseverative	Before 1st correct trial					
Regressive	After 1st correct trial					
Irrelevant	Dig in the irrelevant odor	O3	O3	O3	O3	O3
Old Odor	Dig in the old odor	-	O1	O2	O5	O1
Novel	Dig in the novel odor	O5	-	-	-	-

ANOVA was used unless otherwise noted, with lesion group as the between-subjects factor and the reversal phase as the within-subjects factor. Significance was set at $p<0.05$. *Post-hoc* comparisons were made using Bonferroni post-hoc tests. Welch's correction was applied on t-tests when the groups had unequal variances. One animal was excluded based on external variables that affected on-task performance and confirmed by a Grubbs outlier statistical analysis ($Z=2.29$, $p<0.05$). Analysis and graphing were performed with GraphPad Prism v5.02.

Results

3.1 BLA-lesioned mice are impaired in serial reversal learning

To analyze the role of the BLA in reversal learning, the animal must be able to learn the task. Thus, we first analyzed if the BLA is necessary for acquisition of the 4-choice ODT. BLA-lesioned mice showed no overall difference compared to shams in the number of trials it took to reach criterion (8 out 10 consecutive trials correct, Fig 3C) [$t(20)=1.681$, $p=0.11$] or in the total number of errors made [$t(20)=1.314$, $p=.20$] (Fig 3E). These data are consistent with previous studies showing that the BLA is not necessary to learn an initial cue-reward association (Machado and Bachevalier, 2007; Schoenbaum et al., 2003; Churchwell et al., 2009).

After acquiring the task or after each reversal phase, mice were returned to their homecage. 24 hours later, the mice were placed back in the 4-choice ODT and were required to recall the previous day's cue-reward association before being moved into the relevant reversal phase (Table 1). There was no main effect of lesion [$F(1,20)=0.05$, $p=.82$] or phase [$F(4,80)=1.67$, $p=.16$] on the number of trials to reach criterion during recall across the five days

for either acquisition or reversal phase (Fig 3G). Thus, both sham- and BLA-lesioned animals were capable of recalling the previous day's cue-reward association.

In the first reversal phase (R1), the previously rewarded odor (O1) is now unrewarded, a previously unrewarded odor (O2) is now rewarded, a third odor (O3) remains unrewarded, and a novel odor (O5) is swapped in for O4 (Table 1). In subsequent reversals, no novel odor was introduced. The novel odor was introduced to analyze differences in responding to the two remaining unrewarded cues, one that had never been previously experienced (novel odor, O5) and one that was previously unrewarded (irrelevant odor, O3).

BLA-lesioned mice required significantly more trials to reach criterion than their sham counterparts (Fig 3D). There was a significant main effect of reversal phase [$F(4,80)=11.54$, $p<0.0001$], with both groups requiring less trials to reach criterion across phases. There was a also a significant main effect of lesion [$F(1,20)=7.58$, $p=.01$], with lesioned-mice taking longer to reach criterion than sham mice. There was also a significant interaction effect [$F(4,80)=2.5$, $p=.05$], and Bonferroni *post-hoc* analysis indicated that the BLA-lesioned mice performed significantly worse than sham controls in the R1 and R2 phases (R1: Lesion, 40.85±6.031, Sham 24.22±2.338, $p<0.01$**;** R2**:** Lesion, 40.69±5.127, Sham 24.78±3.550, $p<.05$). These data indicate that BLA-lesioned mice perform significantly worse than the sham-lesioned mice on the first two reversal phases but not on the last three reversal phases.

Taking longer to reach criterion could be due either to making more errors or needing more rewarded trials to learn the new cue-reward association. We first examined the total number of errors made across reversal phases. Both groups made fewer errors as they

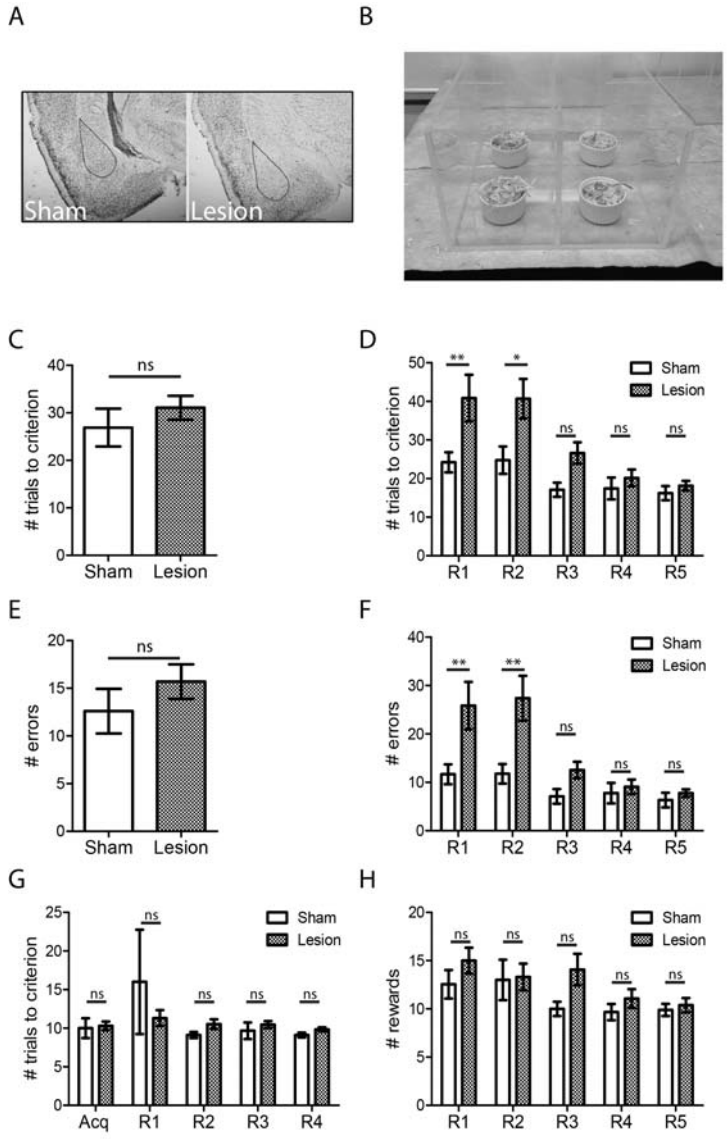

Figure 3. BLA-lesioned mice are impaired at serial reversal learning on a 4-choice odor discrimination task (ODT). (A) Histology sections showing an example sham and lesioned Nissl-stained BLA. Outline is of BLA. **(B)** Image showing the 4-choice ODT set-up. **(C, D)** Total number of trials to reach criterion (8 out of 10 consecutive trials correct) during the acquisition phase (C) and reversal phases (D). There was no significant difference between the two groups during acquisition. BLA-lesioned mice took significantly longer to reach criterion in the first two reversal phases. **(E, F)** Total number of errors made during the acquisition phase (E) and during the reversal phases (F). There was no significant difference between the two groups during acquisition. BLA-lesioned mice made significantly more errors during the first two reversal phases. **(G)** Total number of trials to reach criterion during the recall phase. There was no significant difference between the two groups across phases. **(H)** Total number of rewards accumulated during each of the reversal phases. There was no significant difference between the two groups across phases. N=9 sham, 13 lesion. *, p<0.05, **, p<0.01.

progressed through serial reversal (F(4,80)=10.12, p<0.0001] and lesioned-mice made more errors overall [F(1,20)=9.44, p<0.006] (Fig 3F).

Additionally, there was a significant interaction effect [F(4,80)=3.37, p=0.01]. Post-hoc analysis revealed BLA-lesioned mice made significantly more errors than sham counterparts in

the first two reversal phases (R1: Lesion, *M*=25.85±4.9, Sham, *M*=11.67±2.048, p<0.01; R2: Lesion *M*=27.38±4.626, Sham *M*=9.778±1.793, p<0.01]. These data indicate that BLA-lesioned mice are taking longer to reach criterion in part due to making more errors than their sham counterparts in the first two reversals.

In addition to making more errors, BLA-lesioned mice could also require more rewarded trials to learn the cue-reward association. To determine if this was the case, we analyzed the total number of rewards accumulated for each reversal phase. Though there was a main effect of reversal phase on the number of rewarded trials [F(4,80)=4.21, p=0.004] with the number of rewarded trials decreasing across phases, there was no effect of lesion type on the number of rewards accumulated during each phase (Fig 3H) between the groups [F(1,20)=2.17, p=.16]. Thus, BLA-lesioned mice accumulate the same number of rewards across all reversal phases as their sham counterparts. All together, these data demonstrate that on a 4-choice ODT, BLA-lesioned mice make more errors in earlier reversal phases, which coincides with the requirement of learning a new cue-reward contingency (R1 and R2 phases), but not in later reversal phases which coincide with returning to a cue-reward contingency they have previously learned (R3-R5 phases). Furthermore, they accumulate the same number of rewards as sham-lesioned mice across all reversal phases. This suggests that the number of rewarded trials it takes to learn the new cue-reward association is not affected, but rather inhibiting the response to unrewarded cues is impaired.

3.2 BLA-lesioned mice make more reversal and irrelevant errors in reversal phases where a new cue-reward association is learned

To better understand what kinds of errors BLA-lesioned mice were making during serial reversal learning in phases where a new cue-reward contingency was learned, we analyzed the error profile during phases R1 and R2. In R1, a novel odor was swapped in to replace one of the unrewarded odors, so that there were three types of errors possible – reversal errors (responding to the previously rewarded odor), irrelevant errors (responding to the previously unrewarded odor that is still unrewarded), and novel errors (responding to the novel odor, Table 2). Novel odors were not swapped in for subsequent reversals so as not to act as an unintentional cue informing the animal that the contingencies were being switched. A t-test with Welch's correction (for unequal variances) revealed that BLA-lesioned mice made significantly more reversal errors than sham-lesioned mice (Fig 4A, lesion, 19.62±3.837; sham, 9.000±1.900) [$t(17)=2.479$, $p=0.02$, Welch's correction]. Additionally, BLA-lesioned mice also made significantly more irrelevant errors (Fig 4A, lesion, 4.154±1.154; sham, 1.333±0.4410) [$t(15)=2.283$, $p=0.04$, Welch's correction]. There was no significant difference in the number of novel errors made (Fig 4A, lesion, 2.154±0.5170; sham, 1.333±0.5000) [$t(20)=1.095$, $p=.29$]. Thus, while both groups primarily made more errors to the previously rewarded cue than other errors, BLA-lesioned mice showed a much larger impairment in inhibiting responding to the previously rewarded cue. Additionally, though both groups were unlikely to explore a novel unrewarded odor, BLA-lesioned mice were more likely to respond to a previously unrewarded cue (irrelevant) than sham mice.

During the second reversal phase, no new novel error is introduced, thus the three error types are reversal (responding to the previous day's rewarded odor), old odor (responding to the previously rewarded odor from two phases prior), and irrelevant (responding to the

continually unrewarded odor, Table 2). For the reversal and irrelevant errors, we found a similar pattern among BLA-lesioned mice as in R1, with lesioned mice making more reversal errors (Fig 4C) (lesion, 18.31±3.183; sham, 8.333±1.364) [t(16)=2.880, p=0.01, Welch's correction] and more irrelevant errors (lesion, 2.692±0.7106; sham, 0.7778±0.2778) [t(15)=2.509, p=0.02, Welch's correction]. There was a trend for the BLA-lesioned mice to also make more errors to the old odor, but this was not significant (lesion, 6.385±1.380; sham, 2.667±1.130) [t(20)=1.944, p=.07].

Reversal errors can be broken down into two types of errors: perseverative, returning to the previously rewarded odor before the 1st correct trial, and regressive, returning to the previously rewarded odor after the 1st correct trial. In R1, over 50% of reversal errors were perseverative for both groups of mice. In R2, less than 40% of reversal errors were perseverative for the sham group, but the BLA-lesioned group continued to perseverate (over 50% of reversal errors were perseverative). In R2, BLA-lesioned mice made significantly more perseverative errors in total than sham-lesioned mice (Fig 4E) (lesion, 10.85±2.814; sham, 3.111±0.9044) [t(14)=2.617, p=0.02, Welch's correction]. In contrast, BLA-lesioned mice made significantly more regressive errors in R1 than sham-lesioned mice (Fig 4F) (lesion, 9.077±1.869; sham, 3.889±0.5879) [t(14)=2.786, p=0.01, Welch's correction]. Regressive errors in the sham mice accounted for 46% of reversal errors in R1, and 63% of reversal errors in R2. For BLA-lesioned mice, regressive errors accounted for 46% and 41% of reversal errors respectively for each reversal phase. These data indicate that prior to encountering the correctly rewarded odor, BLA-lesioned mice continue to perseverate on the previously rewarded odor through R2, whereas the sham-lesioned mice increase their exploratory behavior and encounter the

rewarded odor sooner. After the 1st rewarded trial in R2, however, BLA-lesioned mice continue to return to the previously rewarded odor at the same rate as in R1, whereas sham-lesioned mice make more regressive errors compared to perseverative errors. This difference may reflect alternate mechanisms used to learn the task when the BLA is online or off-line. In other words, though the total number of reversal errors for both groups stays approximately the same in both R1 and R2, the BLA-lesioned mice tend to perseverate on the previously rewarded odor before encountering the newly rewarded odor, whereas the sham-lesioned mice more quickly explore the newly rewarded odor, then return to the previously rewarded odor.

To visualize each group's digging behavior given their differences in trial number, we normalized outcome choice to total trial number. sham mice dug significantly more in the rewarded pot than in the previously rewarded pot (rewarded, 53.65±4.657; previously rewarded, 35.25±5.353) [t(16)=2.593, p=.02]. BLA-lesioned mice, however, dug equally in both the previously rewarded pot and the rewarded pot (rewarded, 42.44±4.238; previously rewarded, 41.12±4.851) [t(24)=0.05, p=0.96] (Fig 4B). Furthermore, normalization removed any significant difference between the two groups in terms of the percentage of trials in which they dug in the irrelevant odor. Thus, in R1, BLA-lesioned mice are digging significantly more often in the previously rewarded odor than sham mice.

When the outcome (error type or rewarded trial) was normalized to the number of trials for each subject in R2, sham mice dug significantly more often in the rewarded pot than the previously rewarded pot (Fig 4D) (rewarded, 56.35±5.323; previously rewarded, 32.53±3.431) [t(16)=3.662, p=.002] whereas BLA-lesioned mice did not (rewarded, 39.10±5.799; previously rewarded, 41.53±4.473) [t(24)=0.3314, p=.74]. Again, normalization removed any significant

difference between the two groups in terms of the percentage of trials in which they dug in the irrelevant odor. Thus, BLA-lesioned mice show an impaired ability to inhibit responding to the previously rewarded cue in relation to the rewarded cue, and only during the first two reversal phases.

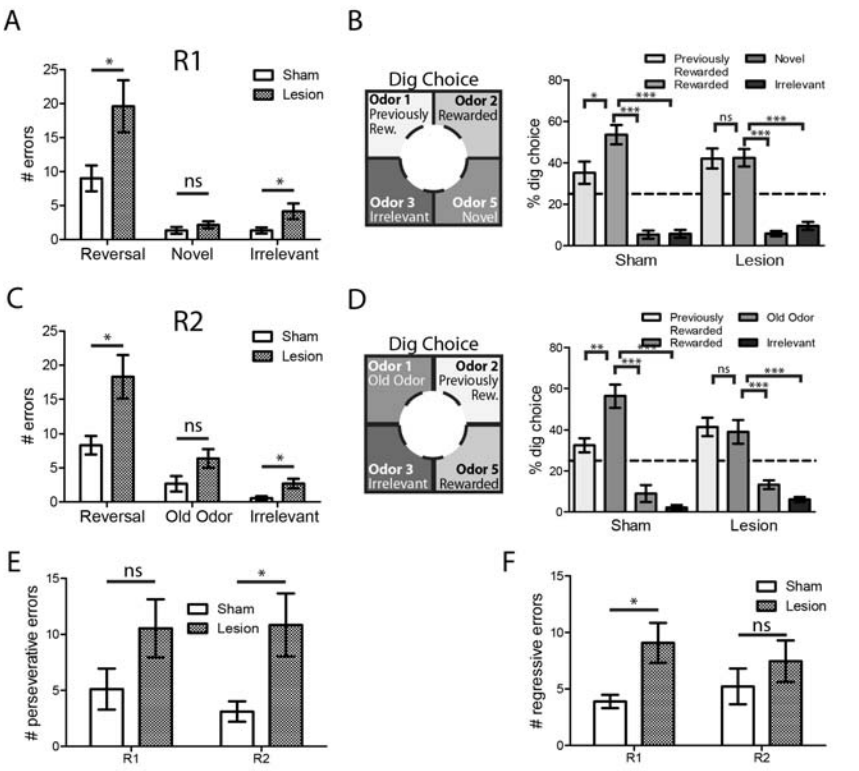

Figure 4. BLA-lesioned mice make significantly more reversal errors during the first two reversal phases. (A,C) Total number of errors made for each error type during R1 (A) and R2 (C). Reversal errors are defined as returning to the previously rewarded odor, novel errors are digging in the new odor, old odor errors are digging in the odor rewarded two phases prior, and irrelevant errors are digging in the continually unrewarded pot. BLA-lesioned mice made significantly more reversal errors than sham-lesioned mice in R1 and R2. **(B, D)** Schematic demonstrating which odors correlate with which dig choice during R1 (B) and R2 (D). Graph on the right depicts percentage of total trials where the mouse chose a particular outcome. Dashed line represents digging at the chance level of 25%. While sham-lesioned mice preferentially dig in the rewarded pot more frequently than the other three pots, BLA-lesioned mice dig equally in the previously rewarded and rewarded pot in both R1 and R2. **(E)** Number of perseverative errors, errors made to previously rewarded odor before the 1st correct trial, made during R1 and R2. BLA-lesioned mice made significantly more perseverative errors than sham-lesioned mice in R2. **(F)** Number of regressive errors, errors made to the previously rewarded odor after the 1st correct trial, made during R1 and R2. BLA-lesioned mice made significantly more regressive errors than sham-lesioned mice in R1. N=9 sham, 13 lesion. ns, not significant; *, p<0.05; **, p<0.01, ***, p<0.0001

3.3 In reversal phases with a new cue-reward contingency, BLA-lesioned mice are impaired at inhibiting responses to the previously rewarded odor, but not at learning the new cue-reward association

In the first two reversal phases where a new cue-reward contingency is learned, BLA-lesioned mice make more reversal errors than sham mice in the first two reversal phases, but accumulate the same number of rewards. This pattern of behavior could result from lower general exploratory activity (e.g., enter fewer quadrants per trial) and therefore less sampling of each odor, from lower exploratory activity on specific trials (e.g., exploring the quadrants in a different pattern on trials with different outcomes), or from a higher rejection rate of the correct odor (e.g., exploring the correct quadrant but proceeding to dig elsewhere).

To address general exploratory activity, we looked at the average number of entries made per trial regardless of outcome. BLA-lesioned mice and sham-lesioned mice showed no significant difference in the average number of entries made per trial either in R1 (Fig 5A) (lesion, 3.971±0.4953; sham, 4.829±0.4829) [t(20)=1.194, p=0.25] or R2 (Fig 5C) (lesion, 3.477±0.2071; sham, 3.609±0.1842) [t(20)=0.4497, p=0.66].

When we looked at the percentage of specific quadrant entries regardless of trial outcome, neither sham nor BLA-lesioned mice showed a strong preference for entering the rewarded quadrant over the previously rewarded quadrant in either R1 or R2 (Fig 5B, D) (R1**:** lesion t(24)=1.375, p=0.18, sham t(16)=1.325, p=.20; R2: lesion t(24)=1.231, p=.23, sham t(16)=0.8613, p=0.40]. However, sham mice showed a preference for entering the rewarded p=0.04] and the irrelevant quadrant (irrelevant, 22.93±0.8232) [t(16)=3.207, p=.006] in R1 (Fig

5B). In contrast, BLA-lesioned mice showed no preference for entering the rewarded quadrant over the novel quadrant [t(24)=0.5624, p=.58], and only a trend of preference over the irrelevant quadrant [t(24)=1.880, p=.07].

In R2, sham mice showed a preference for entering the rewarded quadrant over the old odor quadrant (rewarded, 27.42±1.282; old odor, 24.09±0.9574) [t(16)=2.083, p=0.05] and the

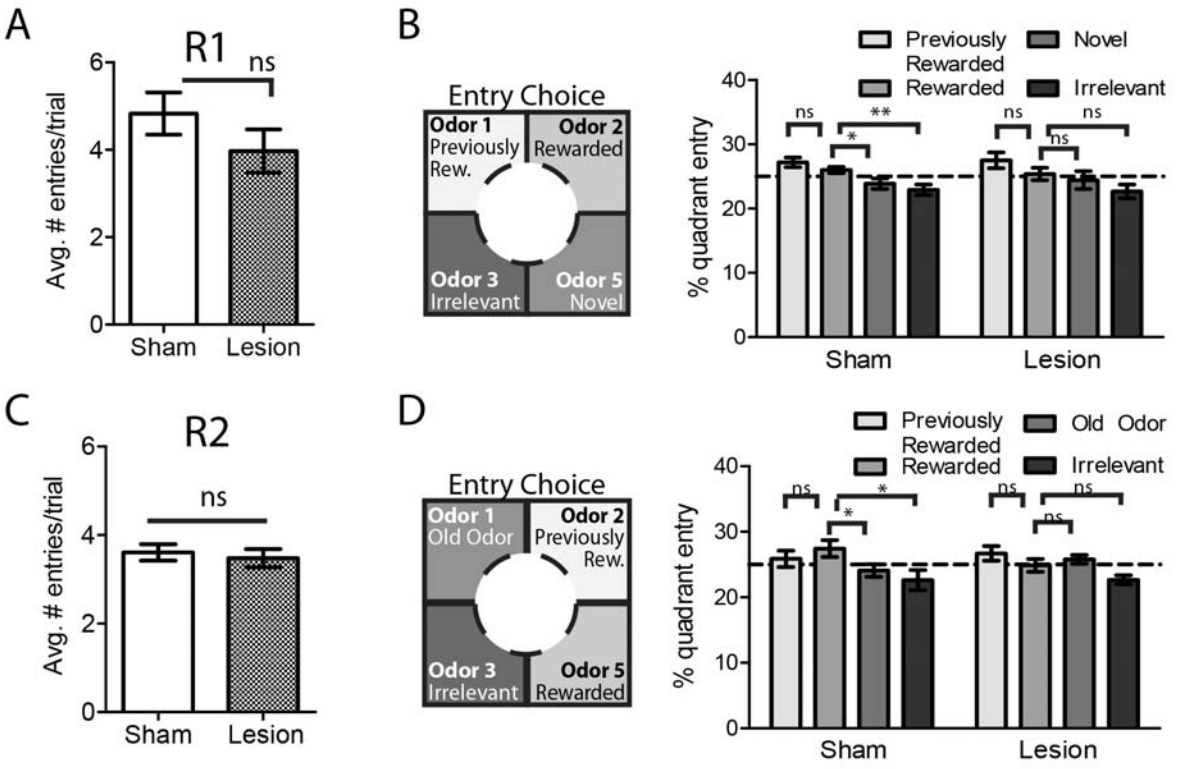

Figure 5. BLA-lesioned mice show same level of general exploratory activity as sham-lesioned mice. (A,C) Average number of entries per trial during R1 (A) and R2 (C). There was no difference between the two groups. **(B, D)** Schematic demonstrating which odors correlate with which quadrants entered during R1 (B) and R2 (D). Graph on the right represents % entries into each quadrant based on odor. Sham-lesioned mice showed a significant difference in entering the rewarded quadrant over the novel, old odor, and irrelevant quadrants in R1 and R2. BLA-lesioned mice showed no preference for entering the rewarded quadrant over any other quadrant. N=9 sham, 13 lesion. ns, not significant; *, p<0.05.

irrelevant quadrant (irrelevant, 22.62±1.524) [t(16)=2.410, p=.03]. BLA-lesioned mice showed no preference for entering the rewarded quadrant over the old odor quadrant [t(24)=0.7673, p=0.45] and only a trend over the irrelevant quadrant [t(24)=1.859, p=0.07]. Therefore, on average, while sham mice may show a preference for the rewarded quadrant over the novel, old odor, or irrelevant quadrants, BLA-lesioned mice are sampling the odors equally. However, this might not be the case if trial outcome is taken into consideration.

To determine if quadrant entry differs between the two groups on trials with a particular outcome, we analyzed the percentage of trials sorted by trial outcome (reversal error, novel error (R1), old odor error (R2), and irrelevant error) that an animal ignored the rewarded quadrant. In other words, we wanted to analyze what percentage of trials resulting in an error did they not sample the rewarded odor. On over half the trials resulting in a reversal error, BLA-lesioned mice did not enter the rewarded quadrant, a significantly higher percentage than sham mice (Fig 6A) (lesion, 55.29±8.541; sham, 31.22±6.916) [t(20)=2.039, p=0.05]. Furthermore, when reversal errors are broken down into its two components – perseverative (errors to the previously rewarded cue before a 1st correct trial) and regressive (errors to the previously rewarded cue after the 1st correct trial) – BLA-lesioned mice had a significantly higher percentage of trials resulting in a regressive error in which they did not explore the rewarded quadrant (Fig 6B) (lesion, 78.05±8.291; sham, 36.24±11.29) [t(19)=3.058, p=.007], but showed no difference for perseverative error trials [t(19)=0.8095, p-0.42]. There were no differences between the two groups on the percentage of trials ultimately ending in a novel error (Fig 6C) or an irrelevant error (Fig 6D) where the animal did not enter the rewarded quadrant [novel: t(14)=0.6121, p=0.55; irrelevant: t(16)=0.006, p=0.99].

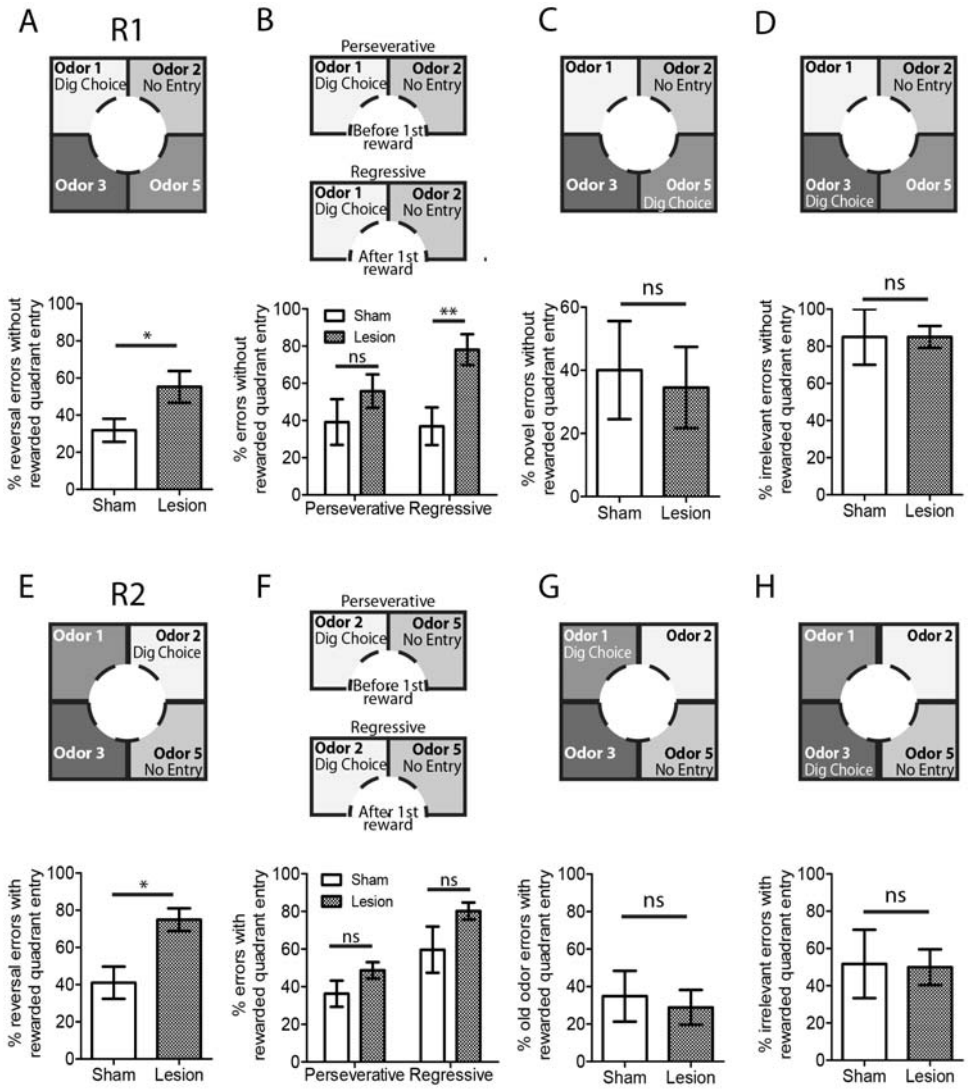

Figure 6. BLA-lesioned mice dig in the previously rewarded odor significantly more on trials when they did not enter the rewarded quadrant. Schematics illustrate behavioral pattern of the mice in R1 (top row) and R2 (bottom). **(A, E)** Percentage of trials where animal did not enter the rewarded quadrant and made a reversal error. On these trials, the BLA-lesioned group was significantly more likely to have not entered the rewarded quadrant. **(B, F)** Schematics illustrate the two components of reversal errors: perseverative errors defined as returning to the previously rewarded odor before the 1st correct trial, and regressive errors, defined as returning to the previously rewarded odor after the 1st correct trial. Graphs depict the percentage of trials where animal did not enter the rewarded quadrant and made a perseverative or regressive error in R1 (B) or R2 (F). On regressive error trials in R1, the BLA-lesioned group was significantly more likely to have not entered the rewarded quadrant. **(C, G)** Percentage of trials where the animal did not enter the rewarded quadrant and made a novel (C) or old odor (G) error. There was no difference between the two groups. **(D, H)** Percentage of trials where the animal did not enter the rewarded quadrant and made an irrelevant error. There was no difference between the two groups. N=9 sham, 13 lesion. *, $p<0.05$, **, $p<0.01$

In R2, the same pattern continues. On almost 80% of trials where the BLA-lesioned mice ultimately made a reversal error, they did not enter the rewarded quadrant. In contrast, sham mice did not enter the rewarded quadrant on less than half of the trials in which they ultimately made a reversal error (Fig 6E) (lesion, 74.96±6.053; sham, 39.87±9.616) [t(20)=3.253, p=0.004]. Furthermore, BLA-lesioned mice did not enter the rewarded quadrant on 80% of the trials that resulted in a regressive error, though this effect is not significant (Fig 6F) [t(19)=1.863, p=0.08]. Again, BLA-lesioned mice had the same percentage of trials as sham-lesioned mice where they did not enter the rewarded quadrant and ultimately made an old odor error (Fig 6G) or an irrelevant error (Fig 6H) (old odor, t(16)=0.0873, p=0.93; irrelevant, t(15)=0.0016, p=0.99]. Therefore, BLA-lesioned mice are more likely to have ignored the rewarded quadrant on trials where they ultimately make a reversal error, particularly after the 1st correct trial, but not on trials when they make any other error.

Another possibility for the high error rate in BLA-lesioned animals is that they show a higher rejection rate for the correct odor, meaning that they explore the correct odor quadrant the same amount as sham mice but ultimately dig elsewhere. We analyzed all the trials in which an entry in the rewarded quadrant was made, and asked what percentage of those trials ended with a dig in any of the unrewarded quadrants. In trials where at least one entry was made into the rewarded quadrant, both groups showed a relatively high rejection rate in both R1 and R2 of 30-40% of trials (Fig 7A). However, there was no significant difference between groups (R1: [t(20)=0.1181, p=0.90], R2: [t(20)=1.041, p=0.31]).

Combined with the previous findings, these data indicate that on average, both groups are sampling each odor approximately equally on any given trial regardless of trial outcome,

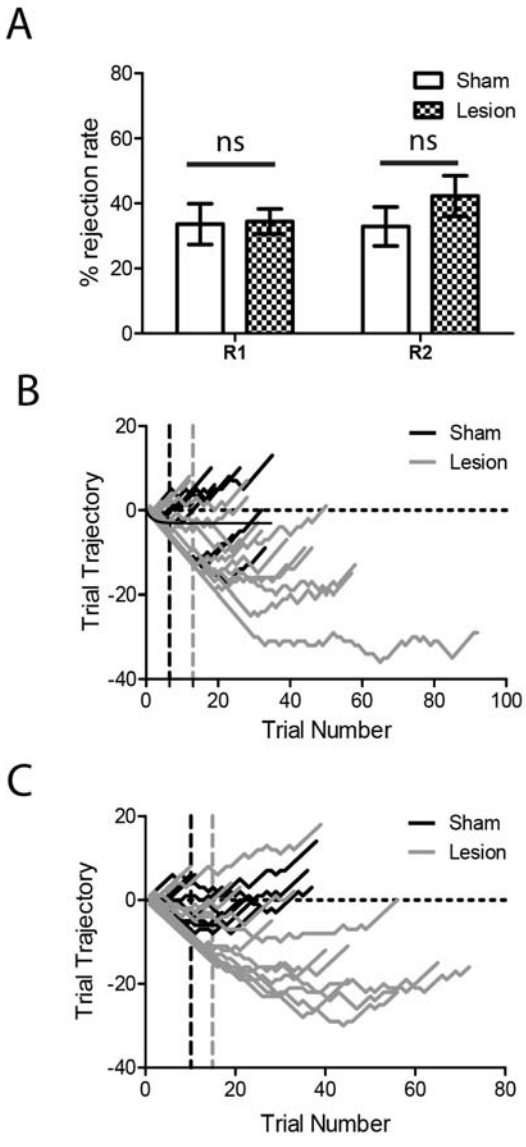

Figure 7. BLA-lesioned mice are not impaired at learning the new cue-reward association. (A) Percent rejection rate, defined as the percentage of trials with a rewarded quadrant entry and a dig in any of the three unrewarded pots. There is no significant difference between the two groups in either R1 or R2. **(B,C)** Analysis of trial trajectory for both groups across trials in R1 (B) and R2 (C). Reversal errors were given a weight of -1, rewarded trials a weight of +1, and other errors a weight of 0. Dotted lines represent average trial to 1st reward for the respective groups. In both R1 and R2, BLA-lesioned mice have a primarily downward slope, even after receiving the 1st reward, indicating that their learning trajectory is weighted primarily by reversal errors as opposed to needing more rewards to learn the new cue-reward association or by making any type of error randomly. N=9 sham, 13 lesion.

though sham mice show a slight preference to explore the rewarded quadrant over the novel, old odor, and irrelevant quadrants. In addition, BLA-lesioned mice and sham-lesioned mice have similar rejection rates after entering the rewarded quadrant (i.e., choosing not to dig in the correct pot after having sampled the correct odor). However, after the first rewarded trial of the new cue-reward association, BLA-lesioned mice were more likely to have ignored the rewarded quadrant (and thus not sampled the correct odor) before making a reversal error

than sham mice. Together, these data suggest that BLA-lesioned mice are impaired at inhibiting responding to the previously rewarded cue rather than being impaired at learning the new cue-reward association.

To further visualize the trial-by-trial learning differences between the two groups, we weighted trial outcome and graphed the trajectory of the learning curve. Rewarded trials were weighted as +1, reversal errors were weighted as -1, and other error types were weighted as 0. Since BLA-lesioned mice made an equal number of rewarded trials as sham but made more errors, we would expect the trajectory to exhibit a downward slope (weighted by -1 trials). Indeed, we found that a majority of the BLA-lesioned mice show a steep downward slope in R1 (Fig 7C) and R2 (Fig 7D). The dotted lines demarcate the average number of trials before getting the 1st reward. Importantly, on average, the sham group shows a positive increase in slope after the first correct trial, whereas the BLA-lesioned group continues to slope downward. This visually demonstrates that the impairment the BLA-lesioned group is showing in R1 and R2 phases is primarily due to making reversal errors instead of making other errors or requiring more trials to learn the new cue-reward association.

3.4 BLA-lesioned mice show no differences in error type when returned to cue-reward contingencies that were previously learned

To determine if BLA lesions affect reversal learning in cases where the cue-reward contingency has already been learned, we analyzed the error profiles across each of the last three phases (Table 2). Though BLA-lesioned mice made more reversal errors than sham-lesioned mice in each of the last three reversals, the effect was not significant (Fig 8A, C, E) [R3: t(20)=1.873,

p=.08; R4: t(20)=1.001,p=.34; R5: t(20)=1.730, p=.10]. There also was no difference in the number of irrelevant errors [R3: t(20)=1.907, p=.07; R4: t(20)=.0855, p=0.93; R5: t(20)=0.1988, p=.84] or old odor errors [R3: t(20)=1.619, p=.12; R4: t(20)=0.7733, p=.44; R5: t(20)=0.2553, p=0.80]. Additionally, there was no significant difference in the number of perseverative errors (Fig 8G) [R3: t(20)=0.8927, p=0.38; R4: t(20)=0.4093, p=0.69; R5: t(20)=0.9633, p=0.35] or regressive errors (Fig 8H) [R3: t(20)=0.1.369, p=0.19; R4: t(20)=1.032, p=0.31; R5: t(20)=1.164, p=0.26] between the two groups in the last three reversal phases (phases R1 and R2 included as comparison). Furthermore, when dig choice was normalized to total number of trials, BLA-lesioned mice preferentially dug in the rewarded pot over the previously rewarded pot in R3 (Fig 8B) (rewarded, 54.65±3.498, previously rewarded, 30.56±3.058) [t(24)=5.185, p<0.0001], similar to sham mice (rewarded, 62.60±6.324, previously rewarded, 25.63±4.990) [t(16)=4.589, p=0.0003]. This effect was consistent through R4 and R5 as well (Fig 8D, F) (*R4:* lesion, t(24)=5.581, p<0.0001 and sham, t(16)=5.066, p=0.0001; *R5:* lesion, t(24)=9.367, p<0.0001 and sham, t(16)=7.170, p<0.0001]. Thus, in later reversal phases coinciding with the subject returning to a previously learned contingency, BLA-lesioned mice do not show any significant impairment in inhibiting responding to the previously rewarded cue, nor do they make significantly more errors.

3.5 BLA-lesioned mice show no gross deficits of entry pattern in reversal phases with a previously learned cue-reward contingency

We next wanted to analyze if there were differences in entry patterns in BLA-lesioned mice compared to sham mice in reversal phases where the cue-reward contingency had previously

been learned. There were no differences in the total number of entries made between sham and lesion mice in any of the reversal phases (Fig 9A, C, E) (R3: t(20)=0.1399, p=0.89; R4: t(20)=1.776, p=0.09; R5: t(20)=0.3651, p=0.72).

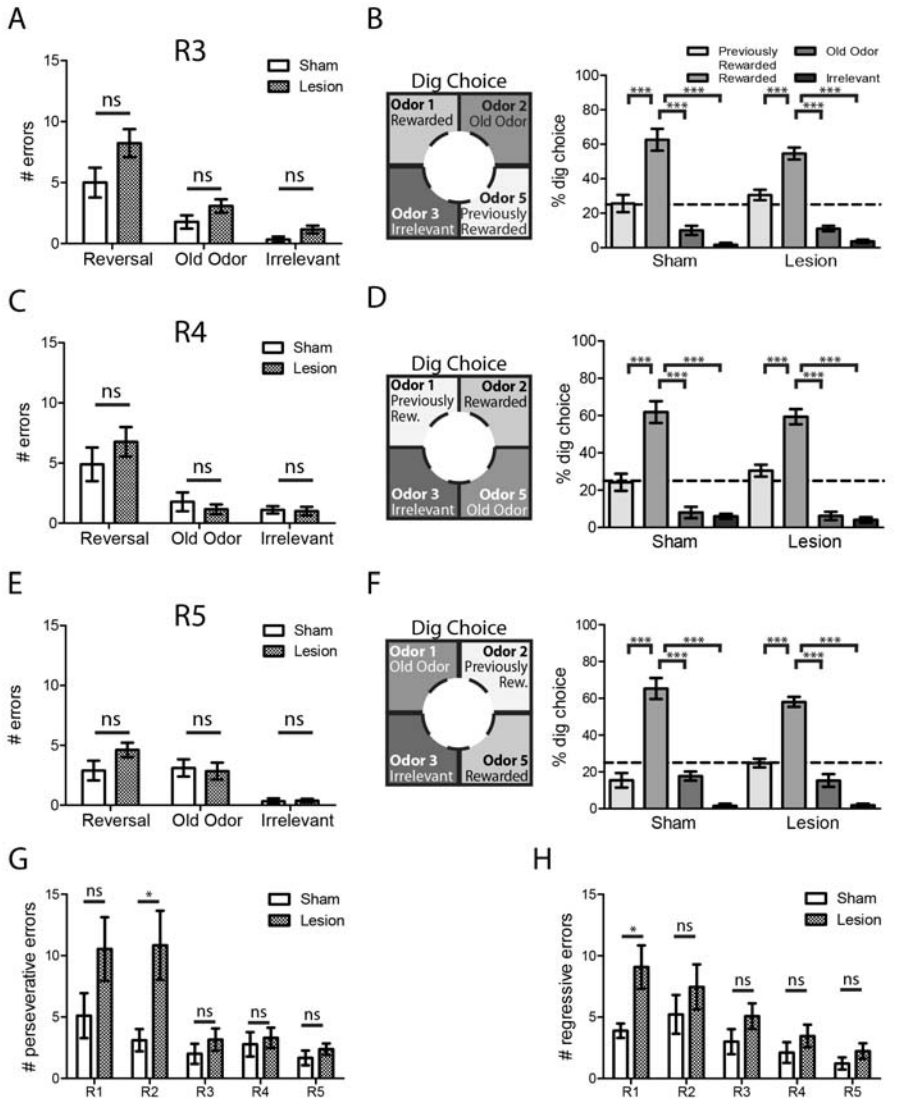

Figure 8. BLA-lesioned mice no longer make more reversal errors during the last three reversal phases. (A, C, E) Total number of errors made for each error type during R3 (A) and R4 (C) and R5 (E). BLA-lesioned mice show no significant difference in the number of each error type made compared to sham-lesioned mice. **(B, D, F)** Schematic demonstrating which odors correlate with which dig choice during R3 (B) and R4 (D) and R5 (F). Graphs on the right depict percentage of total trials where the mouse chose a particular outcome. Dashed line represents digging at the chance level of 25%. Both sham and BLA-lesioned mice preferentially dig in the rewarded pot over the other three pots. **(G)** Number of perseverative errors, errors made to previously rewarded odor before the 1st correct trial, made in all five phases. BLA-lesioned mice made significantly more perseverative errors than sham-lesioned mice in R2 only. **(H)** Number of regressive errors, errors made to the previously rewarded odor after the 1st correct trial, made during all five phases. BLA-lesioned mice made significantly more regressive errors than sham-lesioned mice in R1 only. R1 and R2 are re-shown from Figure 4 for comparative purposes. N=9 sham, 13 lesion. ***, p<0.001

Interestingly, when entries into quadrants are normalized to the total number of entries per trial, sham mice show a significant preference for entering in the rewarded quadrant over the previously rewarded quadrant in R3 and R5, but not R4 (Fig 9B, D, F) (R3*:* $t(16)=3.289$, $p=0.005$; R4*:* $t(16)=0.5379$, $p=0.60$; R5*:* $t(16)=4.243$, $p<0.001$). Additionally, sham mice show a preference for entering the rewarded quadrant over the old odor quadrant in R3, R4, and R5 (R3: $t(16)=3.101$, $p=0.007$; R4: $t(16)=2.593$, $p=0.02$; R5: $t(16)=4.339$, $p<0.001$). Similarly, sham mice show a preference for entering the rewarded quadrant over the irrelevant quadrant in R3 and R5 (R3: $t(16)=3.524$, $p=0.003$; R4: $t(16)=2.311$, $p=0.03$; R5: $t(16)=4.552$, $p<0.001$). In contrast, BLA-lesioned mice never developed a preference for entering the rewarded quadrant over the previously rewarded quadrant (Fig 9B, D, F) (R3: $t(24)=1.528$, $p=0.14$; R4: $t(24)=0.3879$, $p=0.70$; R5: $t(24)=1.767$, $p=0.09$). Furthermore, BLA-lesioned mice only show a preference for the rewarded quadrant over the old odor quadrant in R3 and R4, though there is a trend for preference in R5 (R3: $t(24)=3.073$, $p=0.005$; R4: $t(24)=2.094$, $p=0.05$; R5: $t(24)=1.841$, $p=0.08$). Finally, BLA-lesioned mice show a preference for entering the rewarded quadrant over the irrelevant quadrant in all three reversal phases (R3: $t(24)=5.649$, $p<0.0001$; R4: $t(24)=4.233$, $p<0.001$; R5: $t(24)=3.232$, $p=0.004$). The entry patterns indicate that sham mice develop a preference for entering the rewarded quadrant over the previously rewarded quadrant across the serial reversals, with the preference appearing at R3. In contrast, BLA-lesioned mice continue to show subtle impairments by showing no preference to enter the rewarded quadrant over the previously rewarded quadrant across all reversal phases.

Since BLA-lesioned mice showed particular deficits during R1 and R2 in quadrant entries on trials where they made a regressive error, and they continue to show impairment in

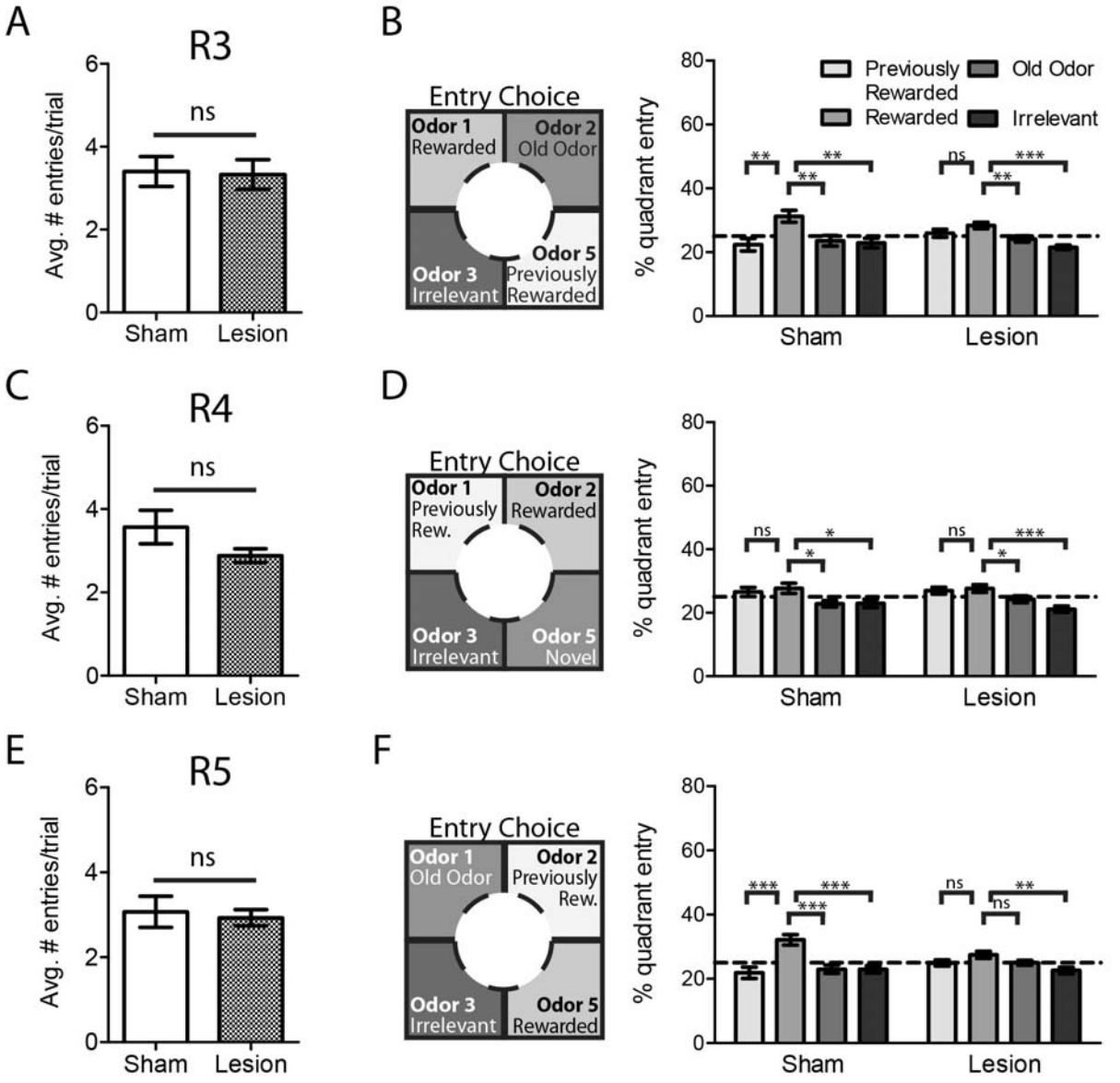

Figure 9. BLA-lesioned mice do not develop a strong preference to enter rewarded quadrant like sham-lesioned mice. (A,C,E) Average number of entries per trial during R3 (A), R4 (C), and R5 (E). There were no differences between the two groups. **(B,D,E)** Schematics demonstrate which odors correlate with which quadrants entered during R3 (B), R4 (D) and R5 (F). Graphs on the right represent % entries into each quadrant regardless of trial outcome. Sham-lesioned mice developed a preference to enter the rewarded quadrant over all other quadrants. BLA-lesioned mice showed no preference for entering the rewarded quadrant over the previously rewarded quadrant, but do develop a preference to enter the rewarded quadrant over the other quadrants. N=9 sham, 13 lesion. ns, not significant; *, p<0.05, **, p<0.01, ***,p<0.001.

preferentially entering the rewarded quadrant in R3-R5, we analyzed their entry pattern into the rewarded quadrant sorted by trial outcome. Interestingly, both sham and BLA-lesioned mice did not enter the rewarded quadrant on a vast majority of trials resulting in a regressive error, but there was no difference between the two groups. Only in R5 did BLA-lesioned mice show a significant difference in the percentage of trials where they made a perseverative error on trials but did not enter the rewarded quadrant (Fig 10J) (lesion, 61.52±8.500; sham, 24.17±15.83) [$t(15)=2.292$, $p=0.04$]. For all other trial outcomes, there was no significant difference.

We then analyzed the rejection rate for the rewarded odor. Both groups showed a much lower rejection rate in the last three reversal phases than in the first two reversal phases, rejecting the rewarded odor only about 20% of the time. There was no difference between the two groups in the rejection rate in any of the three last reversal phases (Fig 11A) (R3: [$t(20)=0.0865$, $p=0.93$]; R4: [$t(20)=0.2749$, $p=0.79$], R5: [$t(20)=0.2160$, $p=0.83$].

Finally, to visualize the trajectory of learning in sham and BLA-lesioned mice, we plotted the learning curve for each of the last three reversal phases as described above. In the last three phases, both groups have a primarily upward slope, indicating that their learning trajectory is weighted by rewarded trials (Fig 11B, C, D). Furthermore, in all three phases, the BLA-lesion group shows similar slopes across phases to that of the sham group, indicating that there is no difference in the learning rate.

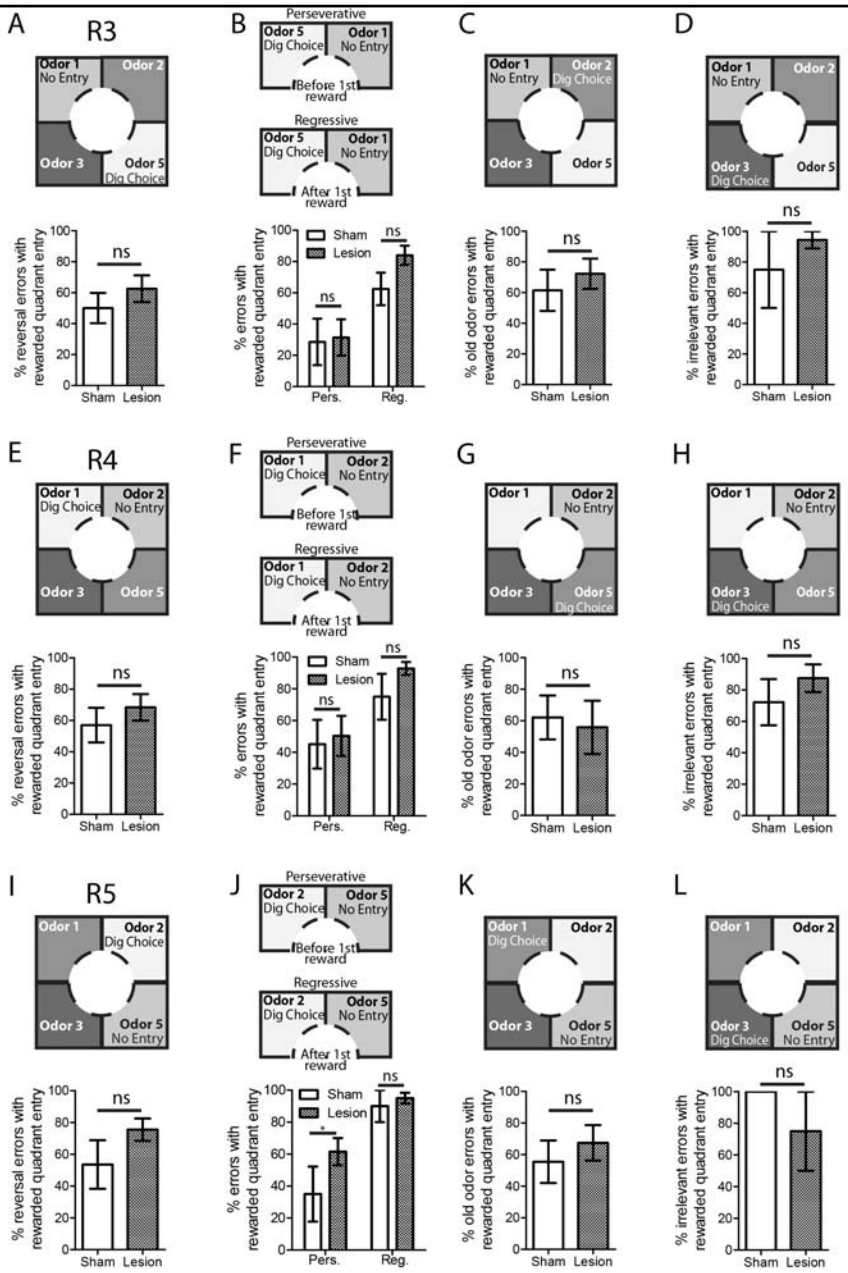

Figure 10. BLA-lesioned mice show no difference in entry patterns during reversal phases where the cue-reward contingencies were previously learned. Schematics illustrate the behavioral pattern of the mice in R3 (top row), R4 (middle row) and R5 (bottom). **(A,E,I)** Percentage of trials where animal did not enter the rewarded quadrant and made a reversal error. There were no differences between groups. **(B,F,J)** Percentage of trials where animal did not enter the rewarded quadrant and made a perseverative or regressive error in R3 (B), R4 (F), or R5 (J). On perseverative error trials in R5, the BLA-lesioned group was significantly more likely to have not entered the rewarded quadrant. **(C,G,K)** Percentage of trials where the animal did not enter the rewarded quadrant and made an old odor error. There was no difference between the two groups. **(D,H,L)** Percentage of trials where the animal did not enter the rewarded quadrant and made an irrelevant error. There was no difference between the two groups. N=9 sham, 13 lesion. *, $p<0.05$.

Discussion

Here we show that excitotoxic lesions of the BLA impair serial reversal learning on a 4-choice ODT. Specifically, animals with BLA lesions were impaired at inhibiting responding to the previously rewarded cue, resulting in a high number of reversal errors. In contrast, the BLA-lesioned mice were not impaired at learning the new cue-reward association, as seen by accumulating the same number of rewards and showing a similar rejection rate of the rewarded odor as sham-lesioned mice. Importantly, the BLA lesions do not affect the capacity for the animal to successfully reverse, but rather slows down the initiation of the process. Interestingly, the impairment in inhibiting responding to the previously rewarded cue was only seen on the first two reversal phases. In the last three reversal phases, the overt effects of the BLA lesions were abolished. However, the BLA-lesioned mice continued to show a difference from the sham-lesioned mice in preference for quadrant entry (not developing a preference to enter the rewarded quadrant over the previously rewarded quadrant), suggesting that subtle impairments may still exist, but that these impairments do not affect the overall rate of reversal.

An advantage of using a 4-choice ODT to address the role of the BLA in reversal learning is the expansion of choices the animal can make on each trial. Interestingly, we found that on the first two reversal phases, BLA-lesioned mice not only made significantly more reversal errors than sham-lesioned mice, but also made significantly more irrelevant errors, and a trend towards making more old odor errors in the second reversal phase. Though the number of errors was greatly reduced compared to the number of reversal errors, this may hint at a

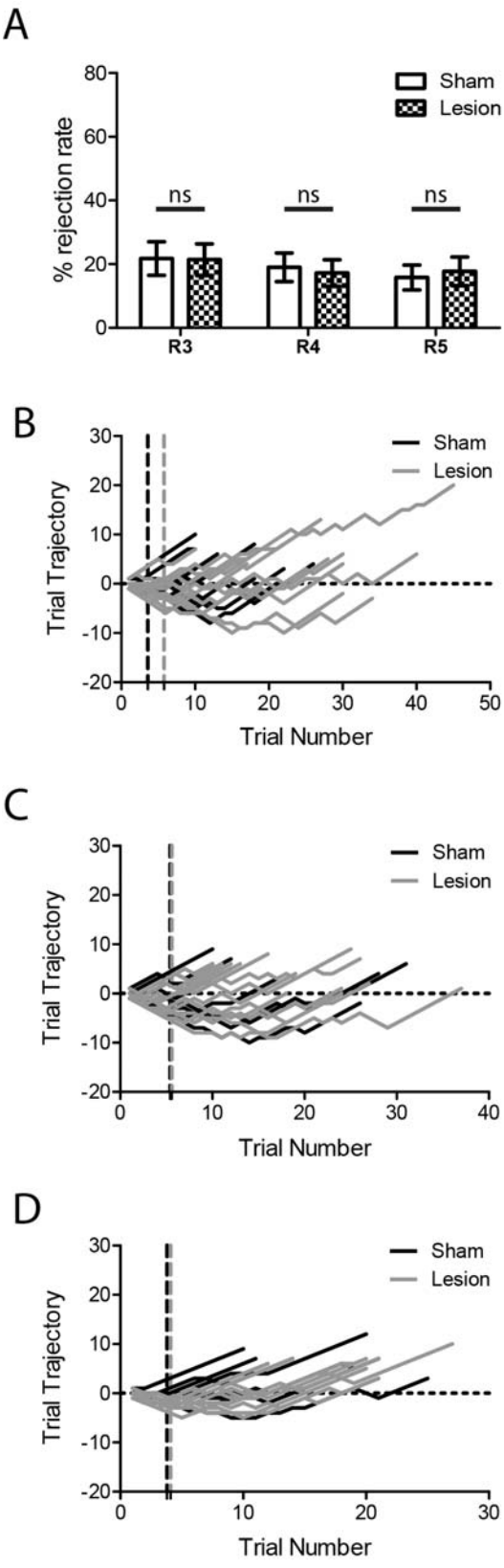

Figure 11. BLA-lesioned mice are not impaired at learning the new cue-reward association during the last three reversal phases. (A) Percent rejection rate. There is no significant difference between the two groups in either R3, R4, or R5. **(B,C,D)** Analysis of trial trajectory for both groups across trials in R3 (B), R4 (C) and R5 (D). In all three reversal phases, BLA-lesioned mice have a primarily upward slope, even after receiving the 1st reward, indicating that their learning trajectory is weighted primarily by rewards. Dotted lines indicate average number of trials to first correct trial. N=9 sham, 13 lesion.

potentially different strategy than the sham-lesioned mice. The sham-lesioned mice only make on average one irrelevant error in either of the first two reversal phases, and only approximately three old odor errors in R2. This may suggest that sham-lesioned mice are quicker to ignore unrewarded odors after minimal sampling than BLA-lesioned mice. However, when the number of errors to each odor was normalized to the total number of trials to reach criterion, there was no significant difference in the percentage of dig choices to the irrelevant odor between sham and BLA-lesioned mice. Thus, it is more likely that the BLA-lesioned animals were significantly impaired in inhibiting responding to the previously rewarded cue and were not actually impaired in initially exploring or subsequently ignoring the other unrewarded cues.

Our findings that BLA lesions impair inhibiting responding to a previously rewarded cue are in line with previous work from our lab and others that neurons in the BLA encode changes in cue or reward value (Tye and Janak, 2007a; Roesch et al., 2010; Zhang et al., 2013; Johnson et al., 2009; Baxter and Murray, 2002). Impairments in encoding either of these changes could result in the impairments we found in our study. Since neurons in the BLA may encode the motivational significance of cues (Tye and Janak, 2007a; Schoenbaum et al., 1999), lesioning the BLA may impair updating a change in the motivational significance of a cue as it switches from being rewarded to not rewarded. Additionally, changes in neuronal signaling in response to changes in reward are thought to act as an attention signal (Roesch et al., 2010). In other words, the firing of BLA neurons encodes a change in the expected reward, but does not signal if the reward size has increased or decreased. Thus, one possibility for the impairments we found is that lesions of the BLA prevent updating of the reward value, and therefore the animal continues to respond for longer to a cue that is no longer rewarding. The nature of our

behavioral task prevents us from differentiating between these two possibilities, and only with time-locked electrophysiological recordings of neurons in the BLA would we be able to determine if the BLA is involved in updating the motivational significance of the cue, changes in reward expectancy, or both.

An additional possibility, and one that is not mutually exclusive from the one described above, is that other regions of the amygdala are involved in guiding the general behavior even when the BLA is offline, thus causing the impaired ability to inhibit responding. In our task, the animal not only has to learn about the cue-reward association (a specific odor predicts a cheerio reward), but also has to learn to approach the cue (approach the pot of scented bedding), and act on the cue (dig in the bedding). In other paradigms, lesions of the BLA do not affect the conditioned approach to the cue, whereas lesions of the central amygdala do (Everitt et al., 1999). Additionally, a more recent model of the amygdala suggests that parallel processing of emotional events occurs within the BLA and central amygdala, with the BLA processing information about specific rewards and associations, and the central amygdala responsible for a more generalized affect towards the event (Balleine and Killcross, 2006). Thus, it is possible that in our 4-choice ODT, the inability to inhibit responding to the previously rewarded odor is in part due to the intact central amygdala promoting an action to the previously rewarded cue without the BLA to modulate that the cue is no longer rewarding.

Finally, though there is no impairment in learning a new cue-reward association either during acquisition or in the first two reversal phases, this does not mean that the BLA does not contribute to these associations, since neuronal firing rates in the BLA change when learning new associations (Schoenbaum et al., 1999; Salzman et al., 2007; Paton et al., 2006). Rather, it

is more likely the case that there is a built-in redundancy of circuits to support learning of new cue-reward associations which can compensate for the lesioned BLA (Balleine and Killcross, 2006; Baxter and Murray, 2002).

In the present study, we found that BLA lesions specifically impaired inhibiting responses to a previously rewarded cue, but did not impair learning a new cue-reward association, in agreement with the role of the BLA in a similar 2-choice ODT (Churchwell et al., 2009). In contrast, in a go/no-go task reversal task set-up, BLA lesions were suggested to impair learning the new cue-reward association (Schoenbaum et al., 2003). There are several possibilities for this discrepancy. First, as previously mentioned, the go/no-go task asks the animal to make a decision based on one cue presentation alone as opposed to making a pairwise comparison, or in this case, a comparison among four choices. These set-ups require different strategies and thus, may reveal differences in learning and behavior. In behaviors where cues are presented simultaneously, a response has to be inhibited to one cue in order to explore another cue. In a go/no-go task, inhibiting responding to one cue does not affect the ability for the animal to respond or inhibit responding to another cue.

Second, though in the latter study it was suggested that BLA lesions impair learning of the new contingencies and do not cause perseveration in responding to the old contingencies, the data itself is collapsed in a manner such that one cannot distinguish between failure to inhibit responding to a previously rewarded cue and initiating responses to a previously unrewarded cue. Additionally, in the 2-choice and 4-choice ODT tasks, one cue is rewarded while the other cues are unrewarded. In the go/no-go task, one cue is rewarding and the other cue is aversive, resulting in the delivery of quinine solution. The salient aversive nature of

quinine may facilitate behavioral responding through other mechanisms than an unexpected omission of reward. Finally, reversal learning on either of the 2-choice or 4-choice ODTs can occur in one session, whereas the go/no-go task takes repeated sessions over days with significant pre-training on the task itself. Thus, there could be significant mechanistic differences in how the reversal learning is supported, such as through memory consolidation or changes in functional connectivity between or within various regions of the brain.

Interestingly, though we found BLA lesions impaired inhibition of responding to the previously rewarded cue, this effect was only seen in the first two reversal phases when a new cue-reward contingency was being learned, whereas previous studies have shown BLA lesions do not affect serial reversal learning (Schoenbaum et al., 2003; Izquierdo and Murray, 2007). In these studies, only two cues were presented such that serial reversals returned to previously learned cue-reward contingencies. One explanation for our findings that BLA lesions did not impair reversal learning in the last three phases is that the contingencies have already been learned and therefore other regions, like the OFC, can compensate by having a previously established state-space (Wilson et al., 2014). In other words, BLA lesions impair learning of a new set of contingencies, but once this learning has occurred, behavioral responding to that set of contingencies can be supported by the OFC. This notion is supported by the timing of neuronal firing in BLA and OFC neurons in a reversal task, with BLA neurons showing selective firing to cues early on, prior to accurate behavioral choices whereas OFC neurons show selective firing to cues later in the task when accurate behavioral choices are consistently occurring (Schoenbaum et al., 1999).

Though it is tempting to conclude from our data that the BLA is necessary to inhibit responding to a previously rewarded cue only in contexts where a new cue-reward association is being learned, an alternative explanation is that lesioned animals simply take longer to learn the "rule of reversal" and only catch up to the sham group by R3, employing the strategy of exploring other cues once they experience the previously rewarded cue as being unrewarded. This potential explanation is supported by the drop in perseverative errors between R2 and R3. The sudden drop in perseverative errors (errors to previously rewarded odor *before* 1st correct trial) in BLA-lesioned mice to similar numbers as in sham mice demonstrate that BLA-lesioned mice are inhibiting responding to the previously rewarded odor and exploring other odors as quickly as the sham mice by R3. Thus, BLA lesions may be delaying higher order rule learning. However, to fully address this entangled issue, future experiments could intersperse previously learned contingencies with new contingencies, for example, by having the rewarded odor switch from O1→O2→O1→O4→O2. If returning to a previous state-space is not affected by BLA lesions but learning about a new state-space is, then we would expect to see impairments in the phases where a new contingency was being learned but not when the contingency was previously learned, regardless of the prior number of reversal phases experienced. So in this example, we would expect BLA lesions to cause deficits in the first and third reversal phases when the animal is switched to O2 and O4 for the first time, but not on the second reversal phase when the animal is returned to O1. In contrast, if BLA-lesioned animals are impaired at learning the "rule of reversal" and simply take longer to learn, then we would not expect to see deficits during later phases of reversal learning regardless if a new cue-reward contingency is being learned. Thus, in this example, we would see deficits in the second reversal phase,

despite being returned to O1, and no deficits in in the third or fourth reversal phases. In this scenario, the more complex 4-choice ODT may reveal the role of the BLA in higher order rule learning in a manner which the 2-choice ODT cannot.

Our study demonstrates for the first time that in a 4-choice ODT, BLA lesions impair inhibiting responding to a previously rewarded cue but do not impair the learning of new cue-reward associations, and that these deficits coincide with when new cue-reward contingencies are being learned. These findings indicate that while other circuits can support learning to respond to a previously unrewarded cue, the BLA is critical to inhibit responding to previously rewarded cues. Furthermore, our data suggest that that the BLA facilitates learning about changes in cue or reward value, particularly in contexts where new contingencies are presented. These impairments were elucidated primarily due to the nature of the behavioral task with the expansion of choice in each phase and the ability to reverse to new contingencies or previously learned contingencies.

Chapter Two

Dorsomedial prefrontal cortex-projecting axons originating from orbitofrontal cortex and basolateral amygdala share similar cortical-like axonal structural characteristics

Abstract

The basolateral amygdala (BLA) acts to guide flexible behavior within a network that includes the orbitofrontal cortex (OFC) and dorsomedial prefrontal cortex (dmPFC). The BLA and OFC both have strong reciprocal connections to the dmPFC. As a region thought to be involved in value encoding, learning, action-outcome encoding, and decision-making, the dmPFC is a prime location to analyze differences in structural characteristics of BLA and OFC axons. Here, we show that limbic-prefrontal cortex axons have a cortical-like structure and are highly similar to intracortical axons from Layers II/III and V neurons that project to Layer I of the barrel cortex. Furthermore, we show for the first time through analysis of bouton turnover that both BLA and OFC axons projecting to the dmPFC are highly stable in adulthood. These data provide a baseline structural comparison and analysis of amygdala and orbitofrontal axons to the dorsomedial prefrontal cortex.

Introduction

The ability to use flexible behavior to adapt to a dynamic, changing environment arises from a network of brain regions sharing information to guide behavior. Along with the amygdala, the orbitofrontal cortex (OFC) and dorsomedial prefrontal cortex (dmPFC) have also been shown to

be necessary for flexible behavior (Kim and Ragozzino, 2005; Johnson and Wilbrecht, 2011; Churchwell et al., 2009).

The BLA and OFC have strong reciprocal connections (Krettek and Price, 1977; Carmichael and Price, 1995a; Öngür and Price, 2000) and work in conjunction to promote flexible behavior (Schoenbaum et al., 2000; Churchwell et al., 2009; Baxter et al., 2000). Humans with lesions of either the OFC or the amygdala show deficits in decision-making, leading to behavior such as inappropriate judgments during a gambling task (Bechara et al., 1999; Gupta et al., 2011; Bechara et al., 1996; Bechara, 2004)**.** Cross-disconnection studies have shown that the BLA and OFC are both necessary for goal-driven behavior (Baxter et al., 2000) or reversal learning (Churchwell et al., 2009).

As previously discussed in chapter one, the BLA is thought to be critical in helping guide flexible behavior by updating cue-reward associations as the reward value changes. The OFC is thought to guide flexible behavior through associative learning of the cue-reward associations as well, but may also contribute to higher order rule learning (Rushworth et al., 2011; Wilson et al., 2014; Cohen et al., 2008) and response inhibition (Horn et al., 2003; Bokura et al., 2001). Furthermore, both regions are involved in encoding outcome expectancies (Schoenbaum et al., 1998). Additionally, one study demonstrated that fMRI tractography values in amygdala-OFC white matters tracts correlated with individuals' scores during reversal learning, and more specifically, that these values correlated with the ability of the individual to learn the higher order rule of reversal learning (Cohen et al., 2008). Thus, these two interconnected regions work in conjunction to guide flexible behavior. Given the similar roles both of these regions play

in flexible behavior, the strong reciprocal connections they share, and the necessity of either region to guide flexible behavior, we were interested in analyzing these regions on a more physiological level to better understand the underlying circuitry of flexible behavior. We chose to analyze the structural characteristics of their axonal projections over time in order to elucidate similarities and differences in structural plasticity of these two regions under baseline conditions.

Using 2-photon *in vivo* imaging, we can analyze the same axons in a mouse every day for several days (Holtmaat et al., 2009). Through this analysis, we can assess the physical characteristics of the axons, such as what types of boutons the axons contain, as well as the rate of bouton formation and elimination (De Paola et al., 2006). The latter analysis has previously been shown through electron microscopy to be representative of synapse formation, elimination, or stability (De Paola et al., 2006; Letizia et al., 2013). Using 2-photon *in vivo* imaging, we can compile structural information of the axons including bouton type, bouton density, survival fraction (how many boutons survive from day one), and turnover rate, all of which will contribute to our knowledge of how the BLA and OFC influence other regions through their connectivity.

2-photon *in* vivo imaging is limited in terms of the depth at which we can image axons without causing severe damage to the brain. Thus, imaging axons *in vivo* either in the OFC or the BLA is not feasible. However, a superficial area also involved in flexible behavior is the dmPFC (Johnson and Wilbrecht, 2011). We chose to analyze axonal projections of the BLA and OFC to the dmPFC for three reasons: 1) The dmPFC is an innervation target for both the BLA and OFC allowing for direct comparisons of structural characteristics across axons to the same

region (Cunningham et al., 2002b; Cavada et al., 2000; Carmichael and Price, 1995a, 1995b); 2) the dmPFC plays a role in flexible behavior tasks such as the 4-chocie ODT described in chapter one (Johnson and Wilbrecht, 2011) and acts as a region that can guide whether the value of a reward merits the effort of action (Rushworth et al., 2004; Floresco and Ghods-Sharifi, 2007); and 3) its superficial location allows us to use 2-photon *in* vivo imaging to analyze axonal structures over days without causing significant damage to the brain. We found that axons from both OFC and BLA to the dmPFC contain primarily *en passant* boutons, have similar densities of boutons, and are highly stable.

Methods

2.1 Animals

C57BL/6 mice were bred in-house and were kept on a 12:12h reverse light-dark cycle with lights on at 10PM. Mice were weaned at post-natal day 21 (P21) and group housed with nesting material. Imaging sessions began at p65 for adults. All animal procedures were approved by the Gallo Center and University of California, Berkeley Institutional Animal Care and Use Committees.

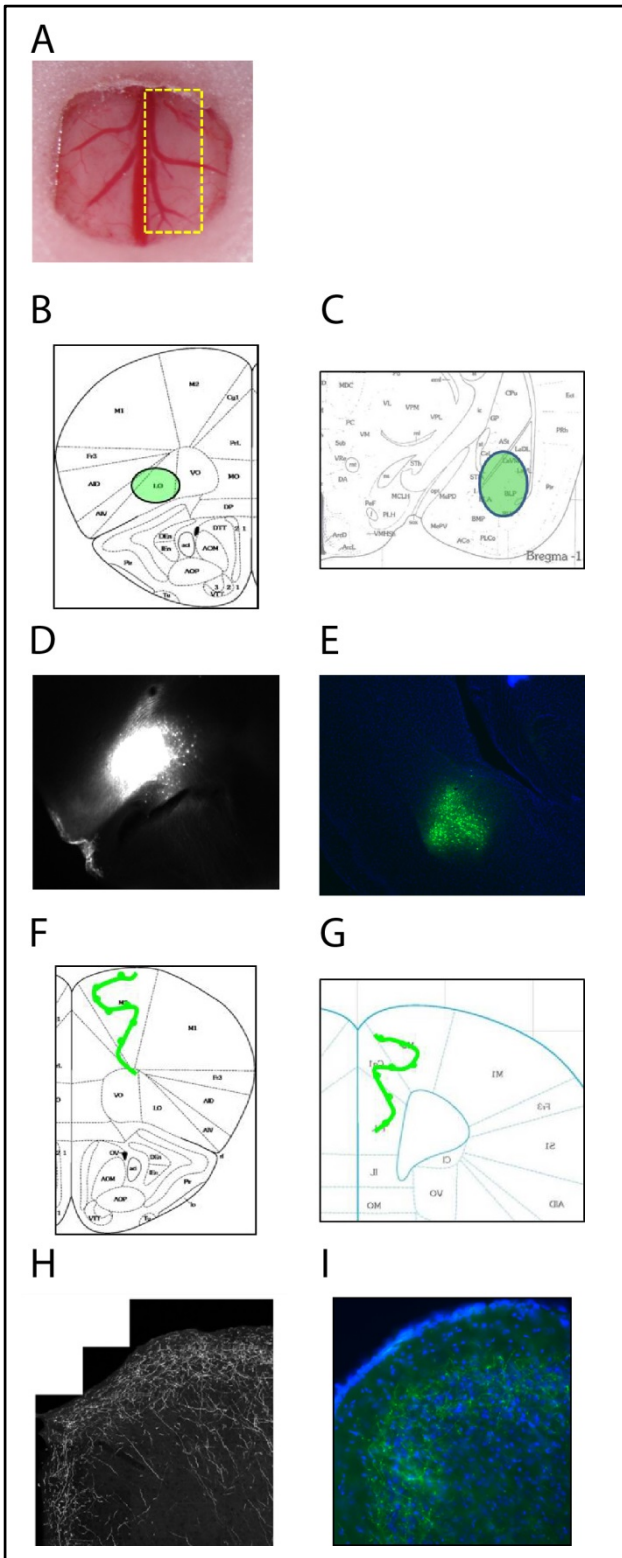

Figure 12. OFC and BLA axons project to different layers of dmPFC (A) An example of a craniotomy and chronic window over the dmPFC. Windows are approximately 3 mm long and 2 mm wide. The yellow box indicates the imaging area over the right hemisphere, approximately 2.5 mm long and 0.8 mm wide. **(B)** Atlas of OFC injection site. Green circle represents site of AAV1/2-CAG-GFP injection into lateral OFC. **(C)** Atlas of BLA injection site. **(D)** Black and white image of virus injection into OFC. **(E)** Virus injection into BLA. Green = GFP fluorescence from virus; Blue = DAPI to label cell nuclei. **(F)** Atlas of OFC axons projecting to Layer I of dmPFC. **(G)** Atlas of BLA axons projecting to Layer II/III of dmPFC. **(H)** Black and white image of OFC axons projecting primarily to Layer I of contralateral dmPFC. **(I)** Image of BLA axons projecting primarily to Layer II/III of ipsilateral dmPFC.

2.2 Virus Injections

Virus injections were made under isoflurane anesthesia using established coordinates (Franklin and Paxinos, 2008) for adults at age p51. Using a Nanoject II injector (Drummond Scientific Company, Broomall, PA), 50 nl of AAV2/1-CAG-GFP was injected in the right (BLA) or left (OFC) hemisphere (BLA: AP: -1.3mm; ML: 3.35mm; V: 4.25mm; OFC: AP: 2.3mm, ML: -1.7mm; V: 2.5mm) (Fig 12B, C, D, E). Prior to surgery, mice were given 50 µl of the NSAID Rimadyl diluted 1:50 with sterile saline (Pfizer, NY, NY)**.** After surgery, mice were given access to 0.5 mg/ml cherry-flavored acetaminophen solution (Perrigo, Allegan, MI) and 0.7 mg/ml oral sulfamethoxazole with 0.1 mg/ml trimethoprim antibiotic solution (Hi-Tech Pharmacal, Amityville, NY) in drinking water.

2.3 Craniotomy Surgery

13 days after virus injection, an ~ 3 mm diameter craniotomy was made over both hemispheres of the dmPFC, as previously described (Holtmaat et al., 2009). Briefly, starting at bregma and moving anteriorly, a window in the skull overlying the dmPFC is removed with a drill, keeping the dura intact. The dura is covered in a small amount of agarose, covered with a coverslip, and sealed with dental acrylic cement (Fig 12A).

2.4 Imaging and Analysis

Beginning 24 hours after surgery, mice were imaged every day up to 8 days under isoflurane anesthesia for up to 120 minutes per session. dmPFC-projecting axons from BLA or OFC neurons were imaged using a Mai Tai HP laser (910 nm, Spectra Physics), Ultima IV in vivo laser-

scanning microscope (Prairie Technologies) and a 40× 0.8 NA objective (Olympus). ~15-30 µM stacks were captured at a resolution of 1084x1084 and a 4X magnification. BLA axons were located approximately ~100 µM below the dura (Layer II/III). OFC axons were located immediately below dura (Layer I).

We used Matlab (MathWorks) and custom axon analysis image software to manually score the boutons as previously described (Fig 13A) (Holtmaat et al., 2009). On average, we analyzed 160.2±7.673 boutons per mouse (BLA: n = 6 mice; OFC: n=8mice) across all imaging sessions. Mice for which we could not analyze a minimum of 95 boutons at day 1 of the experiment were excluded from analysis. All images were scored by an observer blind to the axons' origins.

En passant boutons (EPBs) were identified as previously described (Holtmaat et al., 2009). EPBs were marked as present (1st session) or gained (2nd session or later) if they were 3 times brighter than the axonal backbone and at least 2 µm away from the nearest EPB on either side. EPBs were scored as losses if the brightness dropped below 1.3 times the backbone brightness (Fig 13A). These cutoffs were established by a consortium of imaging labs (Holtmaat et al., 2009).

Seven metrics were used to analyze the structural characteristics of the axons. All measures (except for survival fraction) are averaged across all imaging sessions for the mouse. Survival Fraction – the proportion of boutons present on imaging session 1 that persist to the last day of imaging; Density – the average number of boutons per millimeter axon across all imaging sessions; Boutons gained – the number of boutons gained per millimeter axon on any of the imaging sessions; Boutons lost – the number of boutons lost per millimeter axon on any

of the imaging sessions; Turnover rate – the combined fraction of boutons gained or lost per millimeter axon divided by 2 times the number of boutons present on the first day; Stable boutons gained – the number of boutons gained per millimeter axon that persisted for two or more days; and Stable boutons lost – the number of boutons per millimeter axon that had persisted for two or more days before being lost.

2.5 Histology

All mice were transcardially perfused with 4% paraformaldehyde in PB (0.1M, pH 7.4). Brains were removed and placed in 4% paraformaldehyde overnight. Brains were then transferred to a 0.1M phosphate buffer solution until brains were cut. Coronal sections (100 μM thick) were cut on a vibratome. Sections were mounted and coverslipped with SlowFade (Life Technologies, Carlsbad, CA).

2.6 Statistical analysis

Values are reported as mean (M) ± SEM. Two-tailed t-tests were used for comparison unless otherwise reported. For the survival fraction, an extra sum-of-squares *F* test was used to compare parameters. Significance was set at $p<0.05$. Welch's correction was applied on t-tests when the groups had unequal variances. Analysis and graphing were performed with GraphPad Prism v5.02.

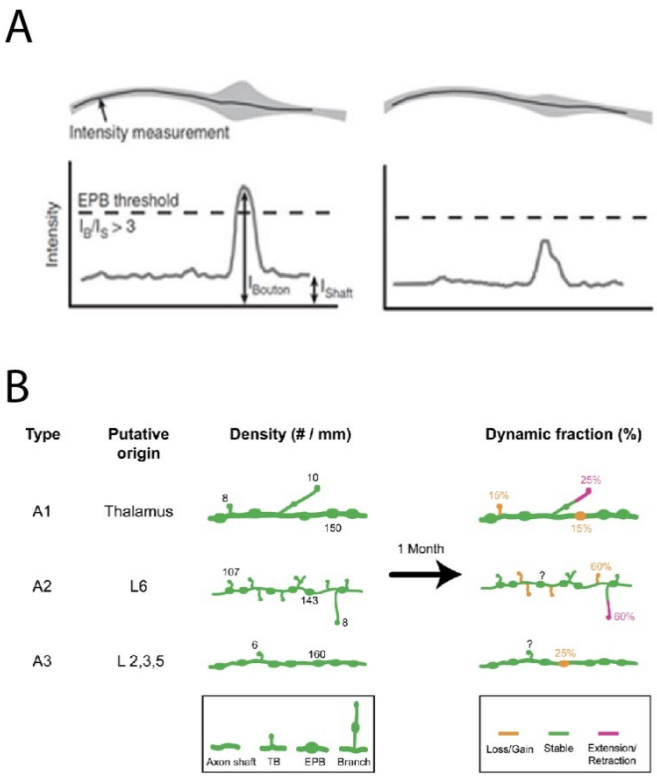

Figure 13. Characteristics and criterion of axons and bouton types (A) *En passant* bouton (EPB) threshold. In order to be counted as an EPB, the varicosity must be at least three times as bright as the backbone. Boutons are counted as lost when the varicosity is no longer 1.3 times as bright as the backbone. Figure adapted from (Holtmaat et al., 2009). **(B)** Three types of axons previously characterized. A1 – thalamocortical axons: thicker axons with EPBs and branches. A2 – L6 cortico-cortical axons: thinnest axons with a majority of highly dynamic *terminaux boutons* (TBs). A3 – L2,3,5 cortico-cortical axons: thin axons with mostly highly stable EPBs. Figure adapted from (De Paola et al., 2006).

Results

3.1 Structural characteristics of OFC- and BLA- dmPFC axons

We used 2-photon laser scanning microscopy to characterize OFC- and BLA-projecting axons in the dmPFC over time through a chronic window in adult mice expressing AAV2/1-CAG-GFP virus. OFC axons were imaged in Layer I of the contralateral dmPFC, while BLA axons were imaged primarily in layers II/III of the ipsilateral dmPFC (Fig 12F, G, H, I), where projections are of highest density, as has been previously described (Bacon et al., 1996; Kita and Kitai, 1990).

Previous *in vivo* imaging studies have characterized axon types into three categories: 1) A1 – thicker axonal branches with large EPBs, numerous branches with a high density of TBs,

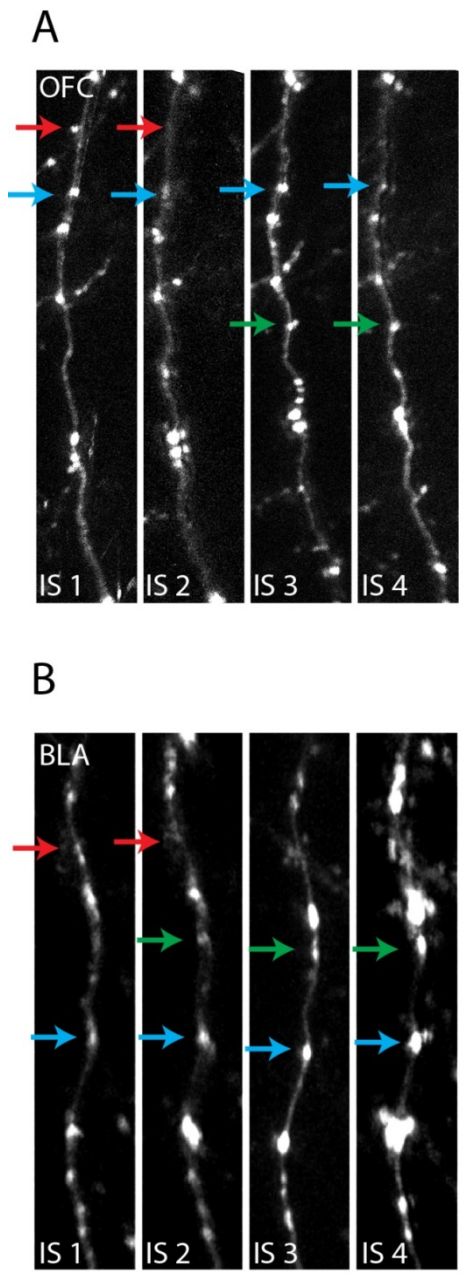

Figure 14. OFC and BLA axons across imaging sessions. (A) OFC axons in dmPFC across imaging sessions over four days. **(B)** BLA axons in dmPFC across imaging sessions over four days. Red arrows, bouton lost. Green arrows, bouton gained. Blue arrows, stable bouton. IS = imaging session.

and 3) A3 – thin axonal branches with a high density of small EPBs and sparse TBs (Fig 13B) (De Paola et al., 2006). Axons originating from the OFC and the BLA both had thin axonal branches and a high density of small en passant boutons with sparse terminaux boutons. (Fig 14A, B).

We first analyzed the average density of boutons per millimeter of axon across days. There was no significant difference in the net density of axonal boutons on any given day from either group (BLA: 178.8±10.25 boutons mm^{-1}, n=7; OFC: 148.4±16.46 boutons mm^{-1}, n=8) [t(13)=1.514, p=0.15] (Fig 15A). These data indicate that both OFC and BLA axons projecting to the dmPFC have structural characteristics similar to A3 type axons previously described (De Paola et al., 2006).

3.2 OFC-and BLA-dmPFC axons have similar bouton turnover rates

OFC and BLA axons may exhibit differences in bouton turnover rate. Turnover rate, or the fraction of boutons gained or lost over a set period of time,

is a measure of synapse formation and elimination (Trachtenberg et al., 2002; Knott et al., 2006; De Paola et al., 2006). We first analyzed daily boutons gained – the number of boutons gained per millimeter between daily imaging sessions and averaged across all sessions per mouse. There was no significant difference in the number of boutons gained (Fig 16A) (BLA: 17.69±2.045, n=7; OFC: 14.88±1.848, n=8) [t(13)=1.021, p=0.33].

We also analyzed boutons lost – the number of boutons lost per millimeter between imaging sessions and averaged across all sessions per mouse. Again, there were no differences between BLA and OFC axons (Fig 16B) (BLA: 9.273±2.089, n=7; OFC: 10.29±1.51, n=8) [t(13)=0.3951, p=0.70].

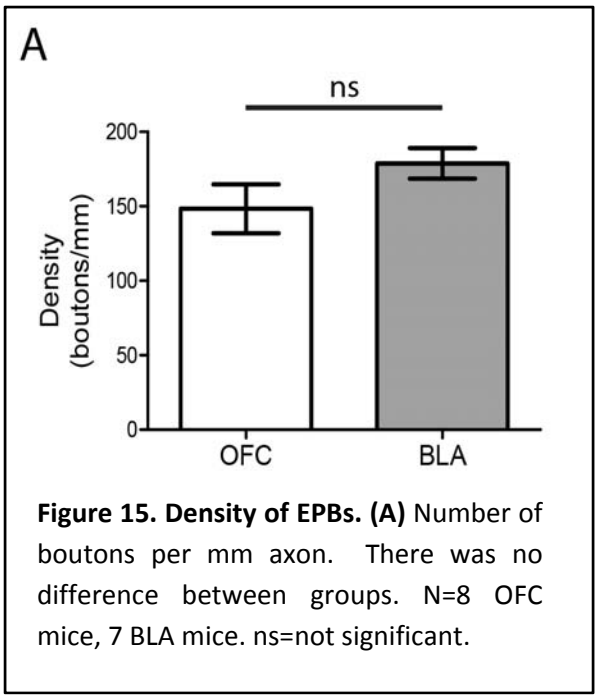

Figure 15. Density of EPBs. (A) Number of boutons per mm axon. There was no difference between groups. N=8 OFC mice, 7 BLA mice. ns=not significant.

Finally, we analyzed the daily turnover rate – the number of boutons gained and lost over two days divided by 2 times the number of boutons present on the first day, averaged across imaging sessions. There was no significant difference between the two groups (Fig 16C) (BLA: 7.762±0.95590, n=7; OFC: 8.574±0.3602, n=8) [t(7)=0.7960, p=0.45, Welch's correction].

To address if all boutons are turning over or if there are two pools of boutons, one dynamic pool and one stable pool, we analyzed the survival fraction of boutons for each group. The survival fraction examines what proportion of boutons which are present on the first day of imaging persist through the last day of imaging. Both groups showed highly stable boutons

across days, with 90% of BLA boutons persisting through Day 4 and 87% of OFC boutons persisting through Day 4 (Fig 16D). There was no significant difference in the survival fraction between groups [F(1,52)=0.1.473, p=0.23]. These data indicate that a majority of boutons from BLA and OFC axons to dmPFC persist for four days or more, with a smaller dynamic pool of boutons. These data are consistent with what is described as a Type A3 axon (De Paola et al., 2006).

3.3 A stable pool of BLA-dmPFC boutons and OFC-dmPFC boutons persist over time

We then examined the number of stable boutons gained – or the number of boutons that were gained and persisted for at least 2 imaging sessions over two days normalized to axon length. We found no differences between groups in the number of stable boutons gained (Fig 17A) (BLA: 11.33±1.047, n=7; OFC: 9.661±1.313, n=8) [t(13)=0.9751, p=0.35).

We next examined the loss of stable boutons over time – or the number of boutons present for at least two days before being lost. Though there was a trend for a lower loss in the number of boutons that had persisted for at least two imaging sessions before being lost in BLA axons, there was no significant difference (Fig 17B) (BLA: 9.044±2.024, n=7; OFC: 19.85±5.532, n=8) [t(8)=1.834, p=0.10, Welch's correction].

Discussion

Here, we show for the first time the dynamics of long range axons in the frontal cortex. We also show for the first time the *in vivo* dynamics of limbic axons in any region. We find that limbic-

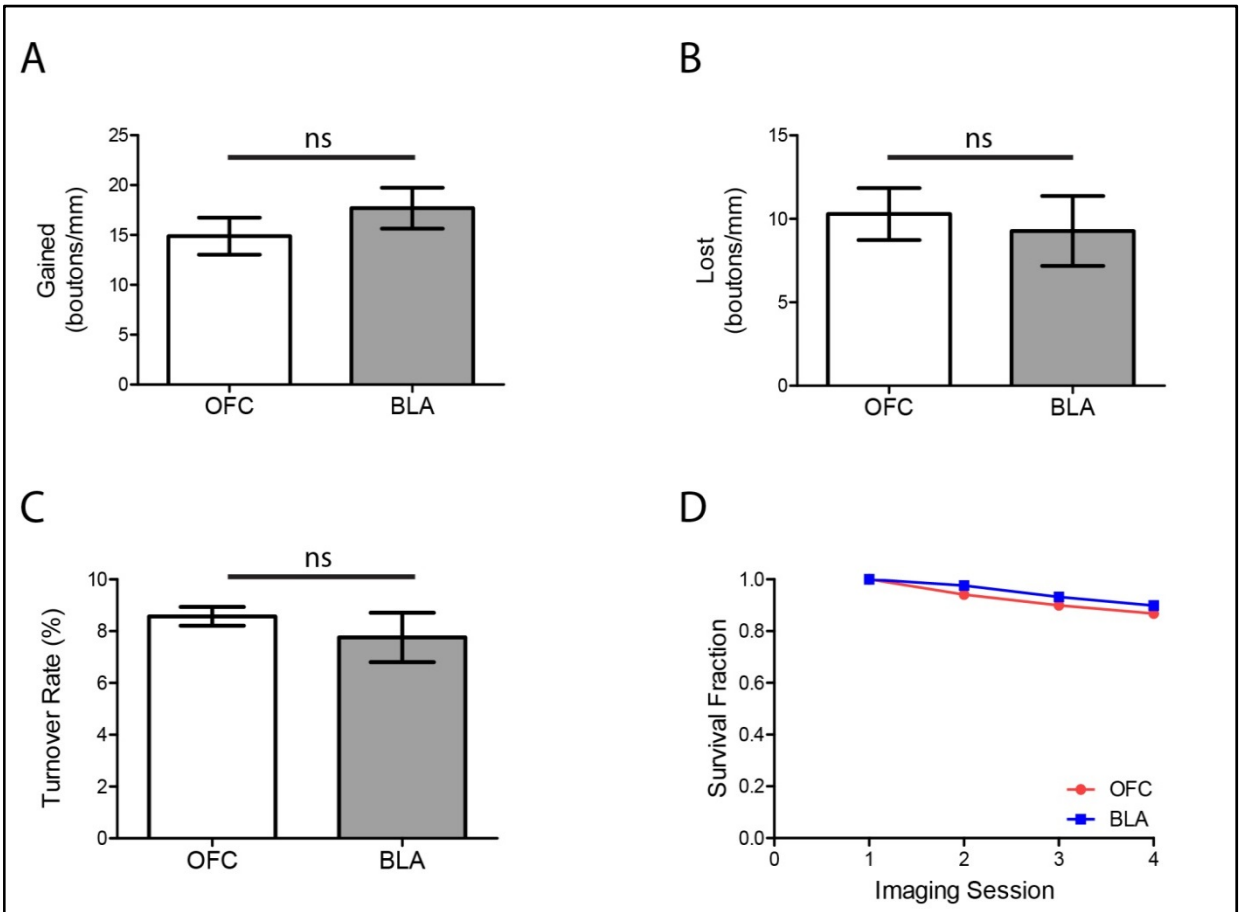

Figure 16. Bouton turnover in OFC and BLA axons (A) Number of boutons gained daily per mm axon averaged across imaging sessions. There was no difference between groups. **(B)** Number boutons lost daily per mm axon averaged across imaging sessions. There was no difference between groups. **(C)** Turnover rate per mm axon, averaged across all imaging sessions. There was no difference between groups. **(D)** Survival fraction. Proportion of boutons present on Imaging Session 1 that persists across all imaging sessions. There was no difference between groups. . N=8 OFC mice, 7 BLA mice. ns=not significant.

cortical axons have similar properties to that of cortico-cortical axons. We demonstrate that axons originating from either the OFC or BLA and which project to the dmPFC are studded primarily by EPBs of small diameter. In addition, both axons from the OFC or BLA to the dmPFC have similar densities of EPBs to cortical axons arising from layers II/III and V, which have

160±50 boutons mm^{-1} (De Paola et al., 2006). Furthermore, the survival fraction over four days is similar to that seen over four days in Type A3 cells (De Paola et al., 2006).

We found no differences between BLA-dmPFC axons and OFC-dmPFC axons in terms of their bouton characteristics. Both sets of axons had similar rate of gains and losses for boutons, resulting in similar turnover rates. Furthermore, both sets of axons had similar survival fractions to that seen in Type A3 neurons (De Paola et al., 2006) and indicates a smaller pool of dynamic boutons alongside a larger pool of highly stable boutons. The similarity of BLA axonal characteristics to OFC axons and other cortico-cortical axons is perhaps unsurprising, since the BLA consists primarily of pyramidal neurons and has previously been described as being cortical-like in terms of its cell composition, afferent synapse targets, and efferent projection targets (Carlsen and Heimer, 1988).

Given the involvement of the BLA in associative learning and the roles of both BLA and dmPFC in emotional, attentional,

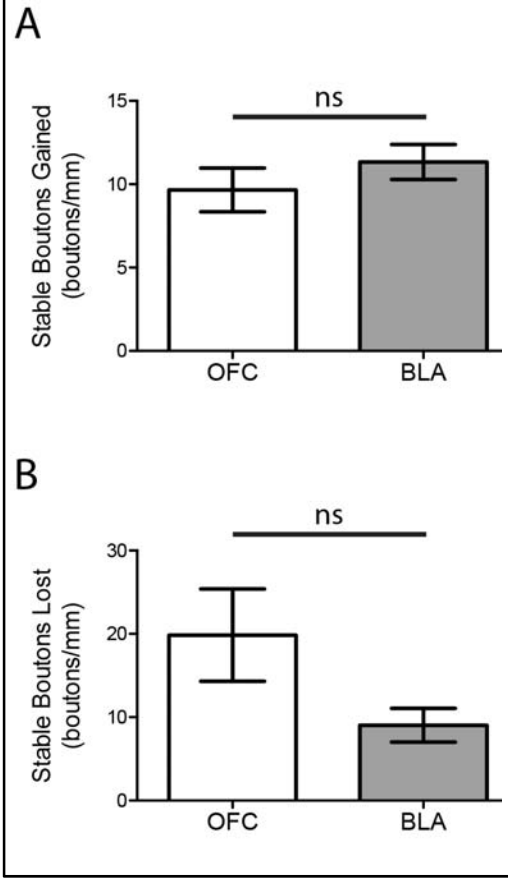

Figure 17. Dynamics of the stable pool of boutons. Number of boutons gained per mm axon over any of the imaging sessions that persist for at least two imaging sessions, averaged across all imaging sessions. There was no difference between groups. **(B)** Number of boutons per mm axon that were present for at least two imaging sessions, then lost over any of the imaging sessions, averaged across imaging sessions. BLA axons had a lower rate of stable boutons lost but the difference was not significant. N=8 OFC mice, 7 BLA mice. ns=not significant.

and motivational aspects of behavior (Devinsky et al., 1995), we might not expect a high rate of turnover in this type of imaging experiment where there are no overt associations with distinct rewards or punishments to be made or goal-driven behavior to guide. Indeed, at the level of cellular activity, the amygdala has a strong inhibitory tone, with activity driven primarily when novel stimuli are associated with a cue (Ledoux, 2004). Therefore, we might expect to see much higher rates of turnover if every day the animal was required to update a cue-reward association as they do in the 4-choice ODT described in chapter one. Unfortunately, these experiments were beyond the scope of this thesis.

Using electron microscopy, EPBs of Type A3 neurons were found to primarily contact dendritic spines (De Paola et al., 2006). This pattern of connectivity has previously been shown in BLA axons projecting to the dmPFC, where the boutons were shown to create asymmetric boutons, 97% of which synapsed onto dendritic spine heads (Bacon et al., 1996). Interestingly, synapses from BLA neurons have been shown to occur both on spines of pyramidal neurons and spiny GABAergic interneurons (Bacon et al., 1996; Cunningham et al., 2008) as well as on dendrites of GABAergic cells (Cunningham et al., 2008). Thus, the BLA likely has direct input onto a variety of cells in the dmPFC.

In sum, this thesis work is pioneering as the first study to use *in vivo* imaging to analyze axonal plasticity over time in neurons originating in either a limbic or frontal region. The findings that baseline EPB plasticity of BLA axons are similar to OFC axons is novel and opens the door for future experiments to analyze the effects of experience-dependent learning on axonal plasticity in either of these regions.

Chapter Three
Conclusions and Future Directions

The work presented in this thesis represents a significant advancement in our understanding of the BLA, both in its role in flexible behavior as well as the characteristics of its connectivity. In the first chapter, we demonstrate that animals with BLA lesions are impaired in their ability to inhibit responding to a previously rewarded cue, indicating a deficit in response inhibition. In the second chapter, we examined the structural plasticity of BLA axons in comparison with OFC axons projecting to the dmPFC. The two sets of axons are highly similar to each other, and highly similar to the previously characterized Type A3 axons of Layer 2/3 and 5 cortical neurons (De Paola et al., 2006). Since each chapter's results are discussed in depth, this chapter will focus on larger implications of the work presented in this thesis as well as future directions.

An animal's survival is dependent on the animal adapting quickly to changes in its environment. Thus, it is disadvantageous for an animal to persist in responding unsuccessfully to an unrewarded cue. Instead, the animal should develop the capacity to inhibit its responses at the appropriate time, while simultaneously directing its attention or behavior elsewhere to retrieve rewards from other areas in the environment.

It is unlikely that the deficits we see in inhibiting responding to the previously rewarded cue after BLA lesions are due to working memory deficits, since the BLA-lesioned animals show far more restraint in responding to either of the other two unrewarded odors than to the one rewarded in the prior session (Fig 4B, C). Thus, it is more likely that the animal is incapable of inhibiting the actual response to the previously rewarded cue. This idea is supported by the

analysis demonstrating that when the BLA-lesioned animals do respond to the previously rewarded cue, they are significantly less likely to have sampled the rewarded odor (Fig 6A, E). Furthermore, when they sample the rewarded cue, the BLA-lesioned mice show equally low rejection rates of the rewarded odor as the sham mice (Fig 7A). This impaired response inhibition is important, especially in the context of diseases where structural abnormalities of the amygdala exist and response inhibition is lacking, such as in ADHD, OCD, and autism (Chambers et al., 2009; Brieber et al., 2007; Szeszko et al., 1999; Agam et al., 2010; Solomon et al., 2009).

Response inhibition is typically thought of as an "executive control" function, arising from the cortices, including the dmPFC and the OFC (Chambers et al., 2009; Horn et al., 2003; Bokura et al., 2001). However, in order to inhibit a response, the animal has to have a preconceived notion of what the outcome will be – in other words, have formed an expectancy of what the cue predicts (Holland and Gallagher, 2004). Both the BLA and the OFC contribute to this outcome expectancy learning, as shown in behavioral tasks such as in the go/no-go task (Schoenbaum et al., 1998). Thus, the BLA, by updating cue-reward associations as they change, may help guide behavioral flexibility by transferring information regarding the association to the OFC and dmPFC so that a correct action can be made, notably inhibiting responding to a no-longer rewarding cue. If the BLA is lesioned, as in our studies or in humans, response inhibition is impaired, resulting in poor decision-making (Bechara et al., 1999; Gupta et al., 2011).

In light of the overlapping roles the OFC and dmPFC play in response inhibition (Chambers et al., 2009; Horn et al., 2003; Bokura et al., 2001) and the roles of the OFC and BLA in guiding outcome expectancies (Schoenbaum et al., 1998), as well as the necessity of all three

regions to successfully complete this task (Johnson and Wilbrecht, 2011; Ragozzino and Rozman, 2007; Kim and Ragozzino, 2005), developing an understanding of how the connectivity among these three regions is affected by flexible behavior would be of great interest. Here, we have laid the foundation for this study by analyzing the structural characteristics of OFC and BLA axons to the dmPFC. Future studies combining this type of analysis in conjunction with the behavior will provide further insight to any similarities or differences seen in these sets of axons, and will further our knowledge of how these three regions interact. Current work with our collaborators has already revealed interesting changes in bouton dynamics as the mice learn the 4-choice ODT, with rule training expanding the size of the pool of dynamic boutons, and bouton turnover correlating with behavioral performance during the acquisition phase of the task (Johnson and Wilbrecht, unpublished data). By adding in the experience-dependent learning component, we may reveal significant differences in the bouton dynamics of OFC and BLA axons.

Though on the surface, the OFC, dmPFC, and BLA have overlapping roles to some extent in guiding flexible behavior, the 4-choice ODT reveals differences in what their precise roles are. Inhibition-mediated inactivation of the dmPFC caused a selective increase in irrelevant errors (Ragozzino and Rozman, 2007) while dmPFC lesion caused only an increase in reversal errors (Johnson and Wilbrecht, unpublished data). Inactivation of the OFC caused an increase in reversal and irrelevant errors (Kim and Ragozzino, 2005), and BLA lesions caused an increase in reversal and irrelevant errors. However, as mentioned in chapter one, when the number of errors of BLA-lesioned mice was normalized to the total number of trials made, the predominant error type was solely reversal errors (% dig choice, Fig 4B, C). These results

suggest that the dmPFC is more likely to be involved in reducing distraction from unrewarding cues or determining if the value of a reward merits an action selection, whereas the BLA is involved in guiding response inhibition through updating cue-reward associations. The OFC seems to play a dual role, simultaneously reducing distraction and promoting response inhibition.

One difference in our study compared to the aforementioned published studies is that we introduced a novel unrewarded odor in the first reversal phase. It would be interesting to see if in the 4-choice ODT, OFC lesion or inhibition also resulted in significantly more novel errors, thus defining its role more broadly to include exploratory behavior towards novel stimuli, as seen in a 2-choice task when a novel stimulus is introduced (Ghods-Sharifi et al., 2008). Beyond understanding the role of each region in flexible behavior, a possibly more interesting dissection would be to understand the role of each pathway. The advent of optogenetics and DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) (Armbruster et al., 2007) will allow future studies to examine the role of each pathway by inhibiting pathway-specific projections during the performance of the 4-choice ODT.

Previous work has demonstrated that juveniles are much quicker to successfully reverse on the 4-choice ODT, and the authors conclude that this may represent a difference in decision-making strategies that regulate behavior appropriate for life stage (Johnson and Wilbrecht, 2011). In humans, amygdala development occurs throughout childhood, reaching its largest volume around the onset of puberty (Uematsu et al., 2012; Ostby et al., 2009; Hu et al., 2013). Furthermore, amygdala activity in response to facial expressions change in children versus adults (Thomas et al., 2001), and the amygdala is hyperactive in adolescents (Malter Cohen et

al., 2013). In rodents, BLA projections to the dmPFC continue to develop and mature through adolescence and into early adulthood (Cunningham et al., 2002a; Verwer et al., 1996) and a similar change in connectivity from the BLA to the dmPFC with age is seen in humans (Gee et al., 2013; Gabard-Durnam et al., 2014). Finally, synaptic plasticity as seen by 2-photon *in vivo* imaging in various parts of the cortex is higher in juveniles or young adolescents than in adults (Zuo et al., 2005; Holtmaat et al., 2005; Grutzendler et al., 2002). Therefore, another interesting facet to explore with regards to the role of the BLA in guiding flexible behavior and its connectivity to the dmPFC would be to repeat the studies described here at different ages, primarily in juveniles versus adults.

The data presented in this thesis set the stage for a more thorough understanding of how the BLA, OFC, and dmPFC interact to guide flexible behavior. Here, we showed that the BLA is necessary for updating cue-reward associations in a 4-choice reversal task, predominately by promoting response inhibition. Furthermore, we show for the first time that, in adults, limbic-frontocortical axons are highly similar to cortico-cortical axons in terms of their structural plasticity. Future studies can further our understanding of these three highly connected regions by looking at pathway-specific contributions to flexible behavior, combining analysis of structural plasticity with behavior, and analyzing age-dependent effects of the BLA on behavior and structural plasticity.

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