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Regulation of Behavioral Flexibility by the Orbitofrontal Cortex and Amygdala

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Regulation of Behavioral Flexibility by the Orbitofrontal Cortex and Amygdala

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An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience
2015

Abstract

Regulation of Behavioral Flexibility by the Orbitofrontal Cortex and Amygdala

By Kelsey Sage Zimmermann

In order to survive in a constantly changing environment, organisms must be able to learn that certain actions or stimuli are predictive of specific outcomes. Equally important is the ability to recognize when a formerly predictive relationship changes and the learned association is no longer relevant or viable. This flexibility in learning facilitates the suppression of previously meaningful behaviors in favor of new, more appropriate responses. The formation of both reward-related and aversion-based associations is known to rely in part on the basolateral amygdala (BLA), which shares rich reciprocal connections with the prefrontal cortex (PFC). The orbitofrontal cortex (OFC) is a highly conserved subregion of the PFC that is necessary for encoding changes to learned associations and facilitating behavioral flexibility. For instance, when an expected outcome is not delivered upon the presentation of a formerly predictive stimulus or the completion of a learned response, the OFC encodes this violation and modifies the previously acquired association accordingly, in part through interactions with the BLA. This process of recognizing changes in contingencies and appropriately changing behavioral responses is an important aspect of goal-directed decision-making. We hypothesize that plasticity within the BLA and the ventrolateral subregion of the OFC (VLO) is necessary for the formation and modification of associative memories, and that functional connectivity between these two regions is critical for goal-directed action selection. This dissertation first reports that activity of Brain-Derived Neurotrophic Factor (BDNF) within the BLA is necessary for both reward-related and fear-based associative conditioning. Next, anatomical and functional connectivity between the VLO and the BLA in mice is described within the context of appetitive instrumental conditioning; here we show that plasticity within the VLO, as well as connectivity between the VLO and the BLA, are necessary for flexible, goal-directed decision-making. Finally, we demonstrate that long-term potentiation in the VLO is necessary for behavioral flexibility in both reward-based action-outcome conditioning and fear-based stimulus-outcome conditioning. Together, these results provide novel insight into how the OFC and the amygdala process information about emotionally salient stimuli in order to mediate associative learning and behavioral flexibility.

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Acknowledgements

I dedicate this thesis to my wonderful parents, Matthew and Cate Zimmermann, who have provided me with endless love and support. In addition to giving me every opportunity in life, they taught me how to think critically and openly about the world, and continue to do so every day. I am deeply and eternally grateful to them, and I feel truly blessed to have been raised by two such amazing people. I would also like to thank Drs. Richard and Dawna Armstrong, incredibly talented and accomplished veterans in the field who nurtured my interest in neuroscience from the time I was 15 and have supported me ever since. For the gifts of their insights, advice, books, and that human brain stem, I will always be extremely thankful.

Emory has been a source of incredible personal and professional support. I'd like to thank the neuroscience community as a whole, and in particular Dr. Yoland Smith, a wonderful rotation advisor and a tireless supporter of students, Drs. Malu Tansey and Victor Faundez, whose enthusiasm and generosity drive the entire program, and Dr. Shawn Hochman, who played no small part in getting me to Emory; I am eternally grateful for his support and encouragement. To my lab mates and the entire floor — it has been a pleasure and a privilege to work (and play) with such amazing, talented people. They have at once been a source of collaboration, support, and friendship, and I count myself lucky to have them in my life. Finally, I'd like to thank Al Dowdle and Ingrid Budreckas, who keep the floor running and work incredibly hard to make our lives easier.

To my wonderful committee members, thank you for the time and effort you devoted to my training; you have been incredibly generous with your expertise and insight, and I count myself extraordinarily lucky to have had the benefit of your guidance. Thank you to Dr.

Geoffrey Schoenbaum, a driving force in the field of orbitofrontal research and the author of many seminal papers that served as much of the basis for my thesis; having the benefit of his experience has been invaluable. Thank you to Dr. Chris Muly, a gifted neuroanatomist who, in spite of having an active clinical presence, still found the time to sit down with me and walk me through my tracing data. Dr. Donald Rainnie has been nothing short of a third mentor to me, and I can't thank him enough for introducing me to techniques and concepts that helped shape my thesis and define my growth as a scientist. Collaborating with him has made me a more conscientious (and argumentative) researcher, and I am exceedingly grateful.

Dr. Kerry Ressler took me in as an undergraduate, and it is no exaggeration to say that he is the reason I am in this program and this area of research. In addition to being an unquestioned leader in the field and a brilliant scientist, Kerry's genuine enthusiasm, constant positivity, baffling energy, and karaoke skills make him a true pleasure and inspiration to work with. I cannot express how grateful I am to him for the influence he has had on my career and my life. Lastly, thank you to Dr. Shannon Gourley. I have had the honor and privilege of being the first graduate student in her lab, and watching the meteoric trajectory of her career in the last few years has been nothing short of awe-inspiring. Shannon is an incredible role model, a dedicated researcher, a tremendously motivating scientist, and a genuinely nice person. I can only hope that my time in her lab has helped me adopt a fraction of her patience, determination, and strength of character. I could not have asked for a better mentor, and I feel extraordinarily fortunate to have been the recipient of her guidance and support.

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Chapter 1:

Introduction

1.1 A Framework and Context for the Dissertation

The following dissertation demonstrates that plasticity in two highly interconnected regions — the orbitofrontal cortex (OFC) and the basolateral amygdala (BLA) — promotes flexible learning and memory in both appetitive and aversive domains. The BLA is a primary site of associative learning; during classical or instrumental conditioning, relevant sensory information converges at this site and encodes the predictive relationship between a given stimulus or action and an outcome. As an association is formed, behavioral responses can be shaped in order to achieve or avoid the outcome, depending on its valence. In a changing environment, an animal must be able to flexibly update these learned contingencies when new information is presented — this process relies largely on the OFC, which can act as an error detector, for example, by registering when a predicted outcome is not delivered as expected. Determining the mechanisms by which these two regions regulate associative learning in both appetitive- and aversive-based tasks will further our understanding of multiple psychiatric disorders that are characterized in part by cognitive or behavioral inflexibility, for instance addiction, obsessive-compulsive disorder, and extinction-resistant phobias.

The formation and modification of outcome-based contingencies relies on learning-dependent plasticity. In order for a stimulus or an action to become associated with an outcome, disparate sensory stimuli, for example an auditory cue and a footshock, converge in the BLA. This establishes the predictive relationship between the stimuli by potentiating the previously weak synaptic strength between inputs from the auditory cortex and responsive neurons in the BLA by pairing the auditory cue with the strongly salient footshock. However, when the contingency changes and the auditory cue no longer predicts a shock, top-down regulation is needed to “de-potentiate” this pathway, either by direct weakening of the connection or by

potentiation of a competing extinction pathway. To explore this mechanism of top-down regulation, we focus on a subregion of the OFC, the ventrolateral orbital cortex (VLO). Again, in order to encode changes to a contingency, mechanisms of learning-dependent neuroplasticity are required in higher-level cortical areas. For the mechanism of neuroplasticity involved in both the formation and modification of associative memories, we chose to explore Brain-Derived Neurotrophic Factor (BDNF), a growth factor largely implicated in learning and memory, as well as activity-dependent neuroplasticity. Our model proposes that ***1)*** production of BDNF within the BLA and activity at its principal receptor, tyrosine kinase receptor B (TrkB), are required for the formation of appetitive- and aversive-based associations (fig.1-1a), and ***2)*** VLO-derived BDNF is necessary for behavioral flexibility following associative learning, possibly by promoting plasticity within the amygdala (fig.1-1b). We further explore the role of learning-dependent plasticity in the VLO in the context of modifying both appetitive- and aversive-based associative memories.

The body of the dissertation will begin in Chapter 2 by examining the role of BDNF activity within the BLA in fear conditioning, as well as cocaine-conditioned place preference (CPP). We find that local viral-mediated knockdown of the *Bdnf* gene, as well as overexpression of a dominant-negative isoform of TrkB, interferes with the acquisition of both fear conditioning and cocaine-CPP. Furthermore, interference with the TrkB receptor also disrupted *extinction* of CPP once the association had been acquired. These data suggest that BDNF-dependent plasticity within the BLA is critical for the formation of both positively-valenced and negatively-valenced associations, and that both production of BDNF and activity at the TrkB receptor are necessary within the BLA for successful associative conditioning.

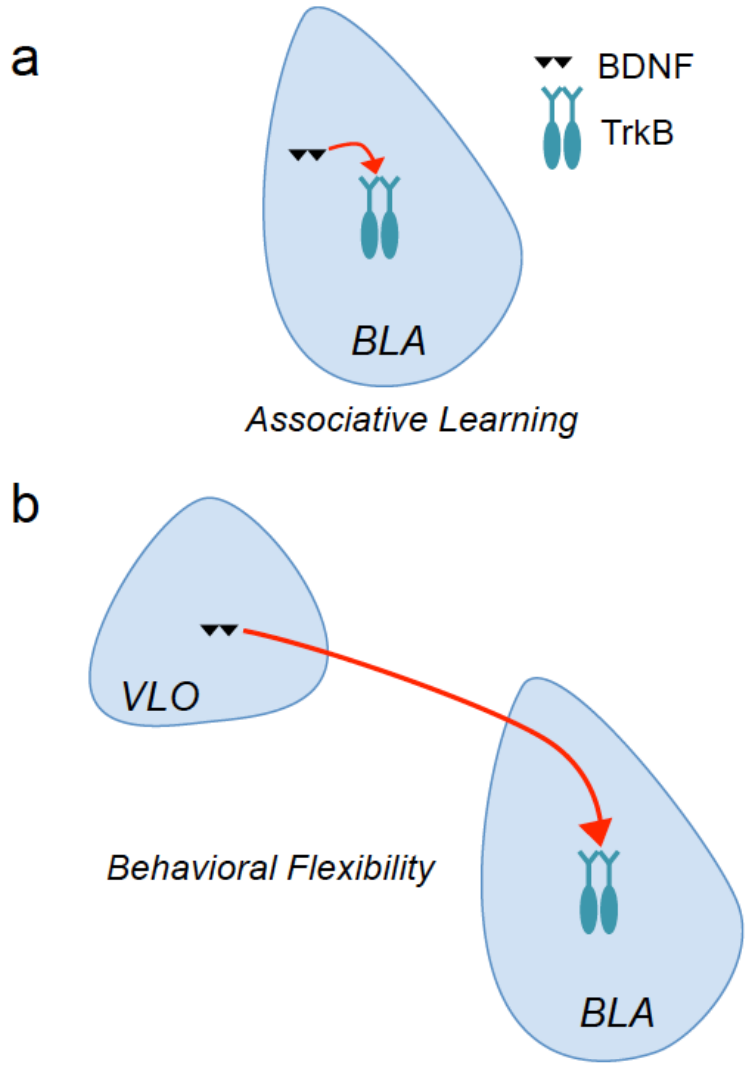
We shift focus in Chapter 3 to the VLO in order to examine how learned associations can be flexibly modified. We first demonstrate that the VLO sends projections to the dorsal striatum as well as the BLA, making it optimally situated to regulate decision-making and associative learning and memory. Next, we show that BDNF-mediated plasticity within the VLO is necessary for flexible, goal-directed decision-making in an instrumental contingency degradation task. Furthermore, we use a modified version of surgical disconnection to demonstrate that connectivity between the VLO and the BLA facilitates goal-directed decision-making in a BDNF-dependent manner. We additionally show that pharmacologically stimulating TrkB can rescue goal-directed response strategies in mice that have been made behaviorally inflexible through ***1)*** overtraining or ***2)*** *Bdnf* knockdown in the VLO. Finally, using Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), we show that activating an inhibitory second messenger cascade in excitatory VLO neurons during instrumental contingency degradation training prevents mice from stably consolidating new information about actions and their outcomes, ultimately causing inflexible, habit-like response strategies.

In Chapter 4, we further utilize DREADD technology and demonstrate the necessity of plasticity within the VLO for behavioral flexibility in both appetitive and aversive paradigms. We replicate and expand on our previous findings that Gi-DREADD-mediated inhibition of VLO neurons prevents stable consolidation of instrumental contingency degradation, ultimately rendering mice less sensitive to changes in the predictive relationship between an action and an outcome. Furthermore, we show that DREADD-mediated inhibition of the VLO during extinction training prevents successful extinction retention following stimulus-outcome fear conditioning, leading to higher levels of stimulus-evoked fear in subsequent tests. Lastly, we report that activating Gi-DREADDs in VLO neurons raises the threshold for long-term

potentiation induction, but does not change intrinsic membrane properties. Together, this evidence suggests that activity-dependent plasticity in the VLO is necessary for consolidating new information that changes or otherwise weakens a previously learned association, and that locally interfering with this process obstructs behavioral flexibility and causes habit-like maintenance of the originally acquired response.

Finally, Chapter 5 synthesizes the findings of the previous three chapters, and discusses the translational importance of our findings, as well as potential future directions for this line of research. Additionally, the role of the OFC in fear-based associative learning and memory is regionally dissected (particularly in light of our focus on the VLO subregion in Chapters 3 and 4) and compared to the known contributions of other prefrontal regions. In this way, we attempt to provide a holistic model of how information about emotionally salient stimuli is processed by cortical regions in order to regulate visceral responses and coordinate behavioral outputs.

Figure 1-1: *BDNF-Dependent Model of Associative Learning and Behavioral Flexibility*



Proposed model. (a) BLA-derived BDNF, as well as local BDNF-TrkB interactions, are both necessary for the formation of appetitive- and aversive-based associative learning (discussed in Chapter 2). (b) VLO-derived BDNF is necessary for behavioral flexibility following associative learning, possibly via anterograde transport to the BLA (discussed in Chapter 3).

Chapter 2:

Bdnf Deletion or TrkB Impairment in the Amygdala Inhibits both Appetitive and Aversive Conditioning

2.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter discusses the role of BDNF-TrkB interactions within the basolateral amygdala in aversive and appetitive conditioning. This work was conceptualized by Drs. Kerry Ressler and Scott Heldt. Research was conducted by Dr. Heldt, the dissertation author, Ms. Kathryn Parker, and Ms. Meriem Gaval, and the document was organized and written by the dissertation author under the guidance of Dr. Ressler. The chapter is reproduced with minor edits from Heldt SA*, Zimmermann KS*, Parker K, Gaval M, Weinshenker D, and Ressler KJ (2014) Bdnf Deletion or TrkB Impairment in Amygdala Inhibits Both Appetitive and Aversive Learning. *Journal of Neuroscience.*

*equal contribution

2.2 Abstract

Brain-derived neurotrophic factor (BDNF) is known to have an integral role in establishing stable memories after learning events. The neuroplasticity induced by Pavlovian fear conditioning has likewise been shown to rely on interactions between BDNF and its principal receptor, tyrosine kinase receptor B (TrkB), in the amygdala after training. Although the necessity of amygdala *Bdnf* expression and TrkB activation for associative learning within aversive contexts has been explored, it is unclear to what extent this interaction is involved in appetitive learning. It is also unclear whether the noted increases in amygdala BDNF after fear conditioning are due to local gene transcription and translation or anterograde transmission from cortical regions. To address both of these questions, we used two lentiviral approaches in mice, using both fear conditioning and cocaine-conditioned place preference (CPP), during acquisition and extinction. First, we suppressed expression of *Bdnf* mRNA in the amygdala of homozygous

floxed mice with a Cre-expressing virus. In a second set of studies, we infused a virus that expressed a dominant-negative TrkB isoform into the same region. These approaches significantly impaired consolidation of fear conditioning and cocaine-CPP, as well as extinction of CPP. Together, these data suggest that BDNF-TrkB signaling is critical for amygdala-dependent learning of both appetitive and aversive emotional memories.

2.3 Introduction

Among its many functions as an influential regulator of neurodevelopment, Brain-derived neurotrophic factor (BDNF) is a well established facilitator of synaptic plasticity within the context of long-term potentiation (LTP) in the adult central nervous system (Korte et al., 1995; Pang et al., 2004; Bramham and Messaoudi, 2005). Interest in the influence of BDNF and its primary receptor, tyrosine kinase receptor B (TrkB), in amygdala-dependent learning has rapidly grown in recent years. Cell bodies in the basolateral amygdala (BLA) are positive for *Bdnf* mRNA, and TrkB is expressed throughout the amygdala (Krause et al., 2008). The BLA is critical for acquiring and consolidating appetitive and aversive learning events, and recent studies have shown that *Bdnf* mRNA is significantly upregulated in this nucleus of the amygdala following Pavlovian fear conditioning (Rattiner et al., 2004; Ou and Gean, 2006).

The role of the BLA in forming associations between conditioned (CS) and unconditioned (US) stimuli has been extensively studied, and the neurocircuitry involved in amygdala-dependent learning is well defined (Davis, 1992; Fanselow and LeDoux, 1999). Fear conditioning induces amygdala LTP, and this plasticity supports the acquisition, consolidation, and expression of aversive associative memories (Rogan et al., 1997; Goosens and Maren, 2002). Furthermore, neural plasticity in the amygdala is dependent on BDNF (Rattiner et al., 2005;

Cowansage et al., 2010), and point mutations at the primary TrkB receptor phosphorylation sites can impair acquisition and consolidation of fear-based learning and memory, as well as synaptic plasticity (Musumeci et al., 2009). Others have found that local infusion of a TrkB antagonist in the amygdala inhibited fear consolidation in rats (Rattiner et al., 2004). Conversely, *in vitro* application of either BDNF or a TrkB agonist lowered the threshold for LTP induction in the BLA (Li et al., 2011, Meis et al., 2012).

Despite ample evidence supporting the necessity of amygdala BDNF-TrkB receptor activity in the formation of fear-based memories, there is a paucity of data addressing the role of this localized neurotrophic activity in other forms of amygdala-dependent learning. In addition to forming associations between conditioned stimuli and aversive outcomes, the BLA is also integrally involved in the acquisition of appetitive contingencies (Everitt et al., 2003). For instance, pretraining excitotoxic lesions of the BLA impair the acquisition of cocaine-conditioned place preference (CPP), while post-training ablation at the same site impairs extinction of the preference (Fuchs et al., 2002) — these data strongly support a regulatory function of the BLA in incentive-based memory formation. Other research has implicated BDNF in drug-seeking behavior (Lu et al., 2004), making the contribution of amygdala BDNF to cocaine-based stimulus-outcome learning a promising area of potential study. The following experiments demonstrate that ***1)*** discrete amygdala manipulations using either Cre-mediated viral knockdown of *Bdnf* or obstruction of BDNF-TrkB activity by infusion of a dominant-negative TrkB isoform (TrkB.t1) impairs acquisition of associative memories, and ***2)*** that the described manipulations similarly affect conditioning in both aversive and appetitive paradigms.

2.4 Materials and Methods

2.4.1 Subjects

All mice were group-housed in standard caging and maintained on a 12-hour light/dark schedule with *ad libitum* access to both food and water. All experiments were run during the light portion of the schedule. Ambient temperature remained at 20°C throughout the experiments. All experiments were approved by Emory University Institutional Animal Care and Use Committee (IACUC) standards with accordance to the Yerkes Primate Research Center regulations.

For experiments involving *Bdnf* knockdown, homozygous *Bdnf*-floxed mice were originally obtained from Jackson Labs (*Bdnftm3Jae*/J; Bar Harbor, ME) and bred within our animal facility. These mice possess loxP sites both upstream and downstream of exon 5 of the *Bdnf* gene. This strain was originated and maintained on a mixed B6, 129S4, BALB/c background and did not display any gross physical or behavioral abnormalities. Experiments were conducted using male mice between 5-10 weeks of age at the start of the experiments.

For experiments involving TrkB inhibition, adult (14-17 weeks old) male C57BL6 mice (Jackson Labs) were used.

2.4.2 Lentiviral (LV) Vectors

LV-Cre: Viral vectors were derived from the HIV based lentivirus backbone pLV-CMV-GFP-U3Nhe, which allows for virally-mediated expression of green-fluorescent protein (GFP) driven by a cytomegalovirus (CMV) promoter. We created a Cre-recombinase expressing viral vector (LV-Cre) by replacing the GFP coding sequence in pLV-CMV-GFP-U3Nhe with the coding sequence for Cre-recombinase. Viral production procedures were described in detail previously (Heldt et al. 2007).

LV-TrkB.t1: Dominant negative TrkB.t1 refers to a truncated version of TrkB that binds BDNF but lacks a catalytic, cytoplasmic kinase domain (Haapasalo et al. 2002). Lentiviral TrkB.t1 transfections inhibit BDNF signaling *in vivo* (Rattiner et al. 2004) and *in vitro* (Li et al. 1998; Haapasalo et al. 2002; Offenhauser, Muzio & Biffo 2002); the virus is tagged with HA for histology purposes. LV-GFP served as a control, leaving the infected neurons intact and expressing the fluorescent marker.

2.4.3 Surgery

Mice received bilateral amygdala microinjections of LV-Cre, LV-TrkB.t1, or LV-GFP. Mice were anesthetized with a Ketastet/Dormitor solution (20% Ketamine, 25% Dormitor, 55% 1xPBS), then mounted in a stereotaxic apparatus. Small holes were drilled in the skull above the injection site, and a Hamilton microsyringe was lowered to the following coordinates from bregma: AP-1.4, ML±3.1 , DV-5.0. A total volume of 1.5 μl was administered at a rate of 0.1 μl/minute (0.2 μl/minute for LV-TrkB.t1). The needle remained in place for 10 minutes after the injection and was removed at a rate of 0.5 mm per minute. The subject's skin was then sewn together using 1.5 metric polyglactin absorbable sutures. After recovery from anesthesia, mice were given a narcotic analgesic, returned to home cages, and monitored daily for 14 days before testing.

2.4.4 Histology

LV-Cre-mediated *Bdnf* knockdown: Mice were deeply anesthetized and brains were rapidly removed and frozen on dry ice. Coronal sections (20 μm) were cut on a cryostat,

mounted on gelatin-coated slides, and stored at -80°C until processed. *In situ* hybridization was performed to examine the expression of mRNA as previously described (Ressler *et al*, 2002).

LV-TrkB.t1: Following ketamine overdose, animals were perfused intracardially using 4% paraformaldehyde in 1xPBS. Sections were incubated with a normal-goat serum blocking solution for one hour at room temperature, followed by anti-HA primary antibody (1:1000) for at least 48 hours at 4ºC and bathed with biotinylated anti-mouse antibody (1:500) for at least 2 hours. These complexes were then amplified with a standard Vectastain Elite ABC kit and stained with diaminobenzidine peroxidase. LV-GFP sections were mounted and counterstained with Hoechst nuclear dye (1:1000).

2.4.5 Behavioral Testing

Conditioned Place Preference

Apparatus: Three-compartment CPP chambers (Med Associates) were used for training and testing.

Pre-test: All animals were pre-tested for individual place preference over a 2 day period. Pre-tests consisted of placing the subject in a neutral (never paired with injection), central compartment and allowing 20 minutes of free access to all 3 chambers. Time spent in each chamber was recorded, and the chamber in which the animal spent the most time was codified as the individual's "preferred side" whereas the other side was its "non-preferred side."

Training: Training utilized a biased design, in which animals received cocaine paired with their non-preferred side. For 3 days, animals received 1 pairing session in the morning and 1 in the afternoon, each lasting 30 minutes. Intraperitoneal (i.p.) injections of cocaine hydrochloride (10 mg/kg, Sigma) were paired with the animal's non-preferred chamber, and i.p.

injections of 1xPBS were paired with the non-preferred chamber. The cocaine pairing was counterbalanced between morning and afternoon to ensure that time of administration did not bias place preference.

Testing: Post-training testing took place 2 days after the completion of training. Animals were allowed to freely explore all three chambers for twenty minutes. Time spent in each chamber was recorded.

Fear Conditioning and Testing

SR-LAB startle response systems (San Diego Instruments) were used for training and testing. During training, mice were placed in the chambers and after 5 minutes received the first of 10 light+shock trials, consisting of a 30 second light CS co-terminating with a footshock. On each of 2 consecutive days after training, conditioned fear was assessed in the same context using the fear-potentiated startle (FPS) paradigm. For each test, each mouse was presented with 4 startle stimuli at each of 3 different startle stimulus intensities (95, 105, 115 dB). After these initial trials, mice were presented with 4 additional startle-alone trials and 4 light+startle trials at each of the 3 startle stimulus intensities. A percentage of FPS was computed for each mouse by dividing the difference between these 2 trial types by the mean startle amplitude on startle-alone trials: percent fear-potentiated startle = (difference/startle-alone)x100.

Elevated Plus Maze

The elevated plus maze consisted of 2 open arms (50 x 6.5 cm) and 2 closed arms with a wall (50 x 6.5 x 15 cm) attached to a common central platform (6.5 x 6.5 cm) to form a cross. The percentage of open arm entries [open arm/(open + closed arm) entries]x100 and percentage time in open arms [time in open arms/(open + closed arms)]x100 were computed.

2.5 Results

2.5.1 Site-specific Bdnf *deletion and TrkB inhibition in the BLA.*

In these studies, we bilaterally infected the amygdala of homozygous 'floxed' *Bdnf* mice with a lentivirus expressing Cre-recombinase, or wildtype mice with a lentivirus expressing TrkB.t1. To determine successful infection and knockdown in the LV-Cre experiments, we performed *in situ* hybridization for *Bdnf* and *Cre-recombinase* expression. We found that *Bdnf* expression in the amygdala was intact following infection with LV-GFP (fig.2-1a), but reduced following LV-Cre infection (fig.2-1b). Spread of the LV-Cre infusion sites was determined via *in situ* hybridization for *Cre-recombinase* (fig.2-1c). For experiments involving TrkB inhibition, mice received amygdalar infusions of LV-GFP (fig.2-1d) or LV-TrkB.t1 tagged with HA (fig.2-1e,f). Spread of the LV-TrkB.t1 virus was determined by visualizing HA; maximal and minimal spread for these experiments is shown in fig.2-1g.

2.5.2 *Knockdown of amygdala* Bdnf *does not affect baseline anxiety.*

We have previously shown that expression of our dominant negative TrkB virus with a truncated cytoplasmic tail (LV-TrkB.t1) in the amygdala impairs fear conditioning and extinction in the rat but that it has no effect on basal measures of anxiety-like behavior (Rattiner et al., 2004; Chhatwal et al., 2006). To likewise determine whether amygdala-specific *Bdnf* deletion affects baseline anxiety, we infected floxed *Bdnf* mice bilaterally with LV-Cre or LV-GFP. Infusions of LV-Cre in the amygdala caused a significant decrease of *Bdnf* at the infusion site [$t_{(26)}$=2.6, p=0.016], but not the hippocampus, used here as a control region (fig.2-2a,b). We found no differences in time in open arms, percentage of open arm entries, or total distance traveled between control GFP-infected mice or those with amygdala *Bdnf* knockdown (fig.2-

2c,d). The regions of the amygdala and hippocampus used for *in situ* analysis are illustrated in fig.2-2e. These data demonstrate that amygdala *Bdnf* knockdown does not affect locomotor behavior or baseline anxiety-like behaviors. Significantly, we have previously shown that LV-Cre infusions into the amygdala of wildtype mice do not produce any changes in motor activity or neuron excitability (Heldt and Ressler, 2010).

2.5.3 Bdnf *knockdown in the BLA impairs FPS.*

We next examined whether *Bdnf* knockdown in the amygdala would result in impaired fear conditioning in the same manner as inhibition of amygdala TrkB function (Rattiner et al., 2004). After bilateral amygdala infection, animals were subjected to 10 light-shock pairings. The next 2 d, they were tested for FPS. We found that *Bdnf*-floxed mice infected with LV-GFP demonstrated significant post-training FPS [fig.2-3a; repeated-measures ANOVA, comparing pre, post1, and post2 percentage FPS within the GFP group, $F_{(2,25)}$=5.28, p=0.01], whereas the mice infected with LV-Cre, with bilateral amygdala *Bdnf* deletion, did not [$F_{(2,25)}$=2.15, p>0.1]. Fig.2-3b demonstrates the extent of LV-Cre infusion in these animals. These data suggest that BDNF must be produced within the amygdala in order for fear-based memories to be established.

2.5.4 Bdnf *knockdown in the BLA and dominant-negative impairment of TrkB in the amygdala delay CPP and impair extinction.*

In addition to the contribution of amygdala BDNF to fear acquisition and consolidation, we also examined the effect of these manipulations on appetitive learning using cocaine-CPP. We found that mice with Cre-mediated knockdown of *Bdnf* in the amygdala failed to show

significant preference for the cocaine-paired chamber over the saline-paired chamber, whereas LV-GFP infected control mice formed a robust preference, spending significantly more time in the cocaine-paired chamber (fig.2-4a). A mixed-model repeated-measures ANOVA was performed, examining a drug (cocaine vs saline chamber) x group (Cre vs GFP) interaction. This was significant for the interaction effect [$F_{(1,27)}$=4.1, p =0.05). Additionally, when we examined the groups separately, we found no significant difference in the Cre group but a significant effect of preference for the cocaine chamber in the GFP group [$F_{(1,14)}$=17.0, p=0.001]. Similarly, mice that received bilateral infusions of the dominant-negative TrkB.t1 virus in the amygdala did not demonstrate preference for the cocaine-paired chamber (fig.2-4b). A mixed-model repeated-measures ANOVA was performed, examining a drug (cocaine vs saline chamber) x group (LV-TrkB.t1 vs LV-GFP) interaction. We found a trend toward a significant interaction [$F_{(1,39)}$=1.99, one-tailed test, p=0.08]. Post hoc analyses revealed that the LV-GFP animals successfully acquired CPP [$t_{(15)}$=2.63, p=0.02], whereas the LV-TrkB.t1 animals did not [$t_{(16)}$=0.96, p=0.35]. Together, these data suggest an influential role for amygdalar BDNF–TrkB interactions in the acquisition of cocaine-CPP.

Finally, these experiments were repeated in an entirely separate cohort of mice to further examine the effect of blocking TrkB in amygdala on CPP extinction (fig.2-4c). A mixed-model repeated measures ANOVA was performed, examining a drug (cocaine vs saline chamber) x group (LV-TrkB.t1 vs LV-GFP) x test (post1 vs post2) interaction. We found a significant chamber x group x test interaction effect [$F_{(1,18)}$=3.36, one-tailed test, p<0.05]. *Post hoc* analyses revealed that, during Test 1, both TrkB.t1 and GFP groups demonstrated a significant place preference. However, after extinction of CPP associated with this first test, the GFP group no longer showed a place preference [$t_{(9)}$=1.07, p=0.3], whereas the TrkB.t1 group continued to

show a preference [$t_{(9)}$ =2.54, p<0.01], suggesting that inhibition of amygdalar TrkB impairs successful extinction of cocaine-CPP.

2.6 Discussion

Synaptic plasticity in the amygdala plays a significant role in encoding representations of both appetitive and aversive learning events. We have demonstrated that either knocking down the expression of *Bdnf* mRNA or sequestering BDNF with an inactive isoform of its principal receptor significantly impairs the consolidation of both aversive and appetitive conditioning. *Bdnf*-floxed mice that received bilateral infusions of LV-Cre into the amygdala failed to show fear-potentiated startle to a conditioned stimulus and did not exhibit significant preference for a cocaine-paired chamber over a saline-paired chamber. Likewise, bilateral infusion of a virus expressing the dominant- negative TrkB isoform, TrkB.t1, produced the same deficits in both tasks. Additionally, to complement prior work showing that LV-TrkB.t1 within amygdala impairs the extinction of conditioned fear (Chhatwal et al., 2006), we also now show that the same manipulation impairs the extinction of an appetitive association. These data suggest that amygdala BDNF is critical for aversive and appetitive conditioning and that activation of amygdala TrkB receptors similarly enables acquisition and/or consolidation of emotionally salient events.

The promotion of synaptic plasticity (Rattiner et al., 2005; Chhatwal et al., 2006; Musumeci et al., 2009; Cowansage et al., 2010) and LTP (Li et al., 2011) in the amygdala via interaction between BDNF and TrkB has been shown to facilitate encoding of the CS-US representation during fear conditioning, and/or enable effective consolidation of the event. When expression or function of either BDNF or its receptor is impeded in the amygdala, behavioral

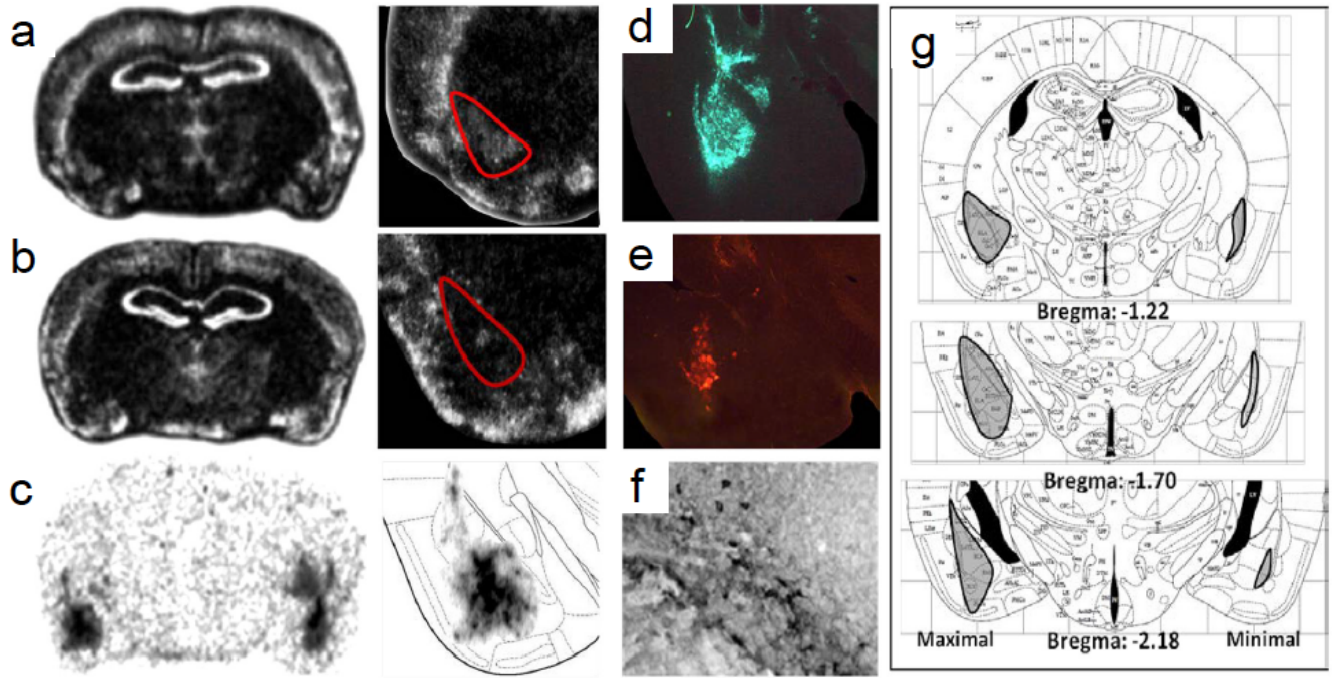
expression of a fear response to the CS in subsequent tests is significantly blunted, which indicates that training of the CS-US contingency was ineffectual. Inhibition of receptor function alone would not exclude the possibility that plasticity was induced principally by prefrontal cortical release of BDNF during acquisition or consolidation. However, when considered alongside the results from local knockdown of the *Bdnf* gene, these data suggest that production of BDNF within the amygdala is also necessary for a lasting memory of the association to be established. Whether BDNF produced within the BLA is acting solely on local TrkB receptors in the BLA and/or central amygdala in a largely isolated amygdalar circuit or being transported to other downstream targets as well is still unknown. In the current experiments, *Bdnf* knockdown in the amygdala would affect BDNF release at terminals in other regions, whereas the effects of TrkB.t1 overexpression would be more locally confined. It is therefore important to consider that, although the effects of TrkB inhibition are specific to the infected region, knockdown of *Bdnf* in the amygdala potentially affects multiple behaviorally relevant postsynaptic target sites that receive BDNF via transmission from the BLA.

Although our infusions spanned multiple amygdalar nuclei, *Bdnf* mRNA is absent from the CeA and expression is most prominent in the BLA (Krause et al., 2008). Some, but not all, of our infusions extended beyond the amygdala to surrounding cortical areas that are positive for *Bdnf* mRNA (e.g., endopiriform nulei and, less frequently, the piriform cortex), but infection was not strong in these areas and did not seem to affect behavioral outcomes. We therefore suggest that knockdown of *Bdnf* in the BLA, rather than other amygdalar nuclei or surrounding cortical areas, most likely generated behavioral differences. In contrast, TrkB is present throughout the amygdala and is also known to be expressed in glia (Kumar et al., 1993; Zhou et al., 1993); therefore, we cannot state, based on our data, which region of the amygdala is most reliant on

TrkB activation for associative learning or whether expression of the virus in white matter tracts like the external capsule contributed to the behavioral differences.

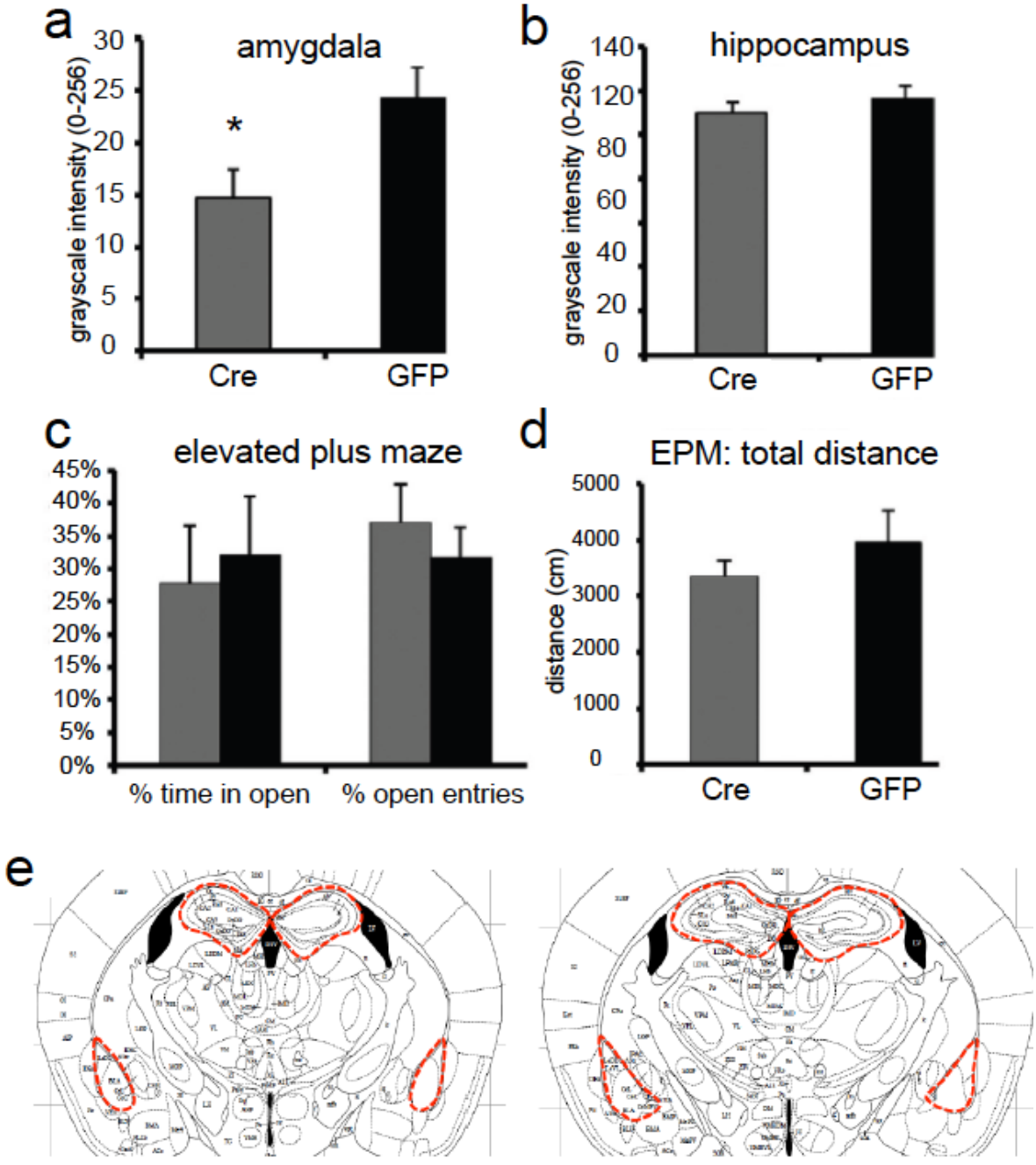
Despite the opposing valences of fear- and incentive-based learning, there are clear advantages to studying the two motivations in concert. The lateral and basolateral nuclei of the amygdala have long been acknowledged as primary sites of associative learning in fear conditioning, but it is also widely understood that the amygdala codifies information about positively as well as negatively valenced stimuli. What is ostensibly an evolutionary advantage (having one structure that encodes a variety of emotionally disparate events) also potentially represents a risk for developing comorbid pathologies. For instance, in humans, the val66met *BDNF* gene variant has been associated with increased risk for substance abuse (Cheng et al., 2005) as well as impaired fear extinction (Frielingsdorf et al., 2010; Lonsdorf et al., 2010; Soliman et al., 2010). This same variant has been shown to affect amygdalar activity during emotional memory formation (van Wingen et al., 2010, Soliman et al., 2010) and can also confer a risk for developing disorders that are based upon opposite motivations (i.e., addiction and post-traumatic stress disorder). There are many shared components in the neurocircuitry underlying aversive and appetitive learning, and exploring the extent to which these pathways overlap will permit a more comprehensive approach to studying healthy and maladaptive consolidation processes that are used in forming memories of emotionally relevant events.

Figure 2-1: *Site-specific* Bdnf *knockdown and TrkB dominant negative inhibition with lentiviral vector approaches.*



(a) Image showing *Bdnf* in situ hybridization of the amygdala following LV-GFP injection or (b) following LV-Cre injection. (c) Qualitative image showing Cre-recombinase in situ hybridization of the amygdala following LV-Cre injection. (d) Lentiviral infection with LV-GFP into BLA (4x). (e) Lentiviral infection with LV-GFP into BLA (20x). (f) LV-TrkB.t1 visualized with an anti-HA antibody recognizing the HA epitope tag at the C-terminal tail of the truncated TrkB (20x). (g) Infusion maps for LV-TrkB.t1, imposed on representative sections from Paxinos and Franklin (2001) *Mouse Brain Atlas*, for the maximal (left) and minimal (right) spread of LV-TrkB.t1 viral infusions. The actual infusions were bilateral, with no difference in left versus right infusion volumes.

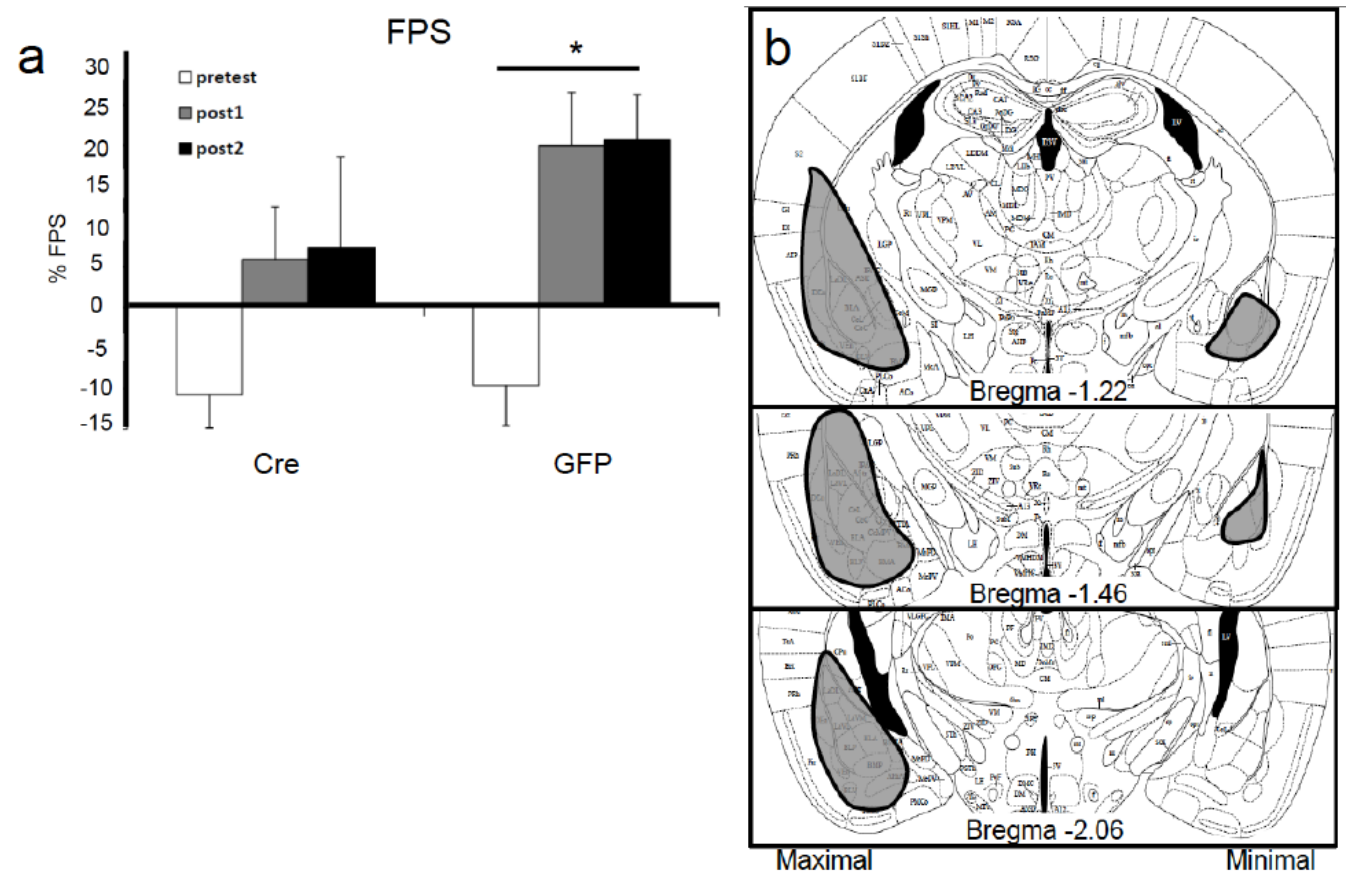
Figure 2-2: *Knockdown of* Bdnf *is specific to the infused region and is not associated with general anxiety-like effects.*



Relative *Bdnf* mRNA expression in amygdala (a) and CA3 region of dorsal hippocampus (b) of mice injected with LV-Cre or LV-GFP in the amygdala. *Bdnf* expression was significantly reduced at the site of infusion, but was not affected in the non-infused control region. (c) LV-Cre

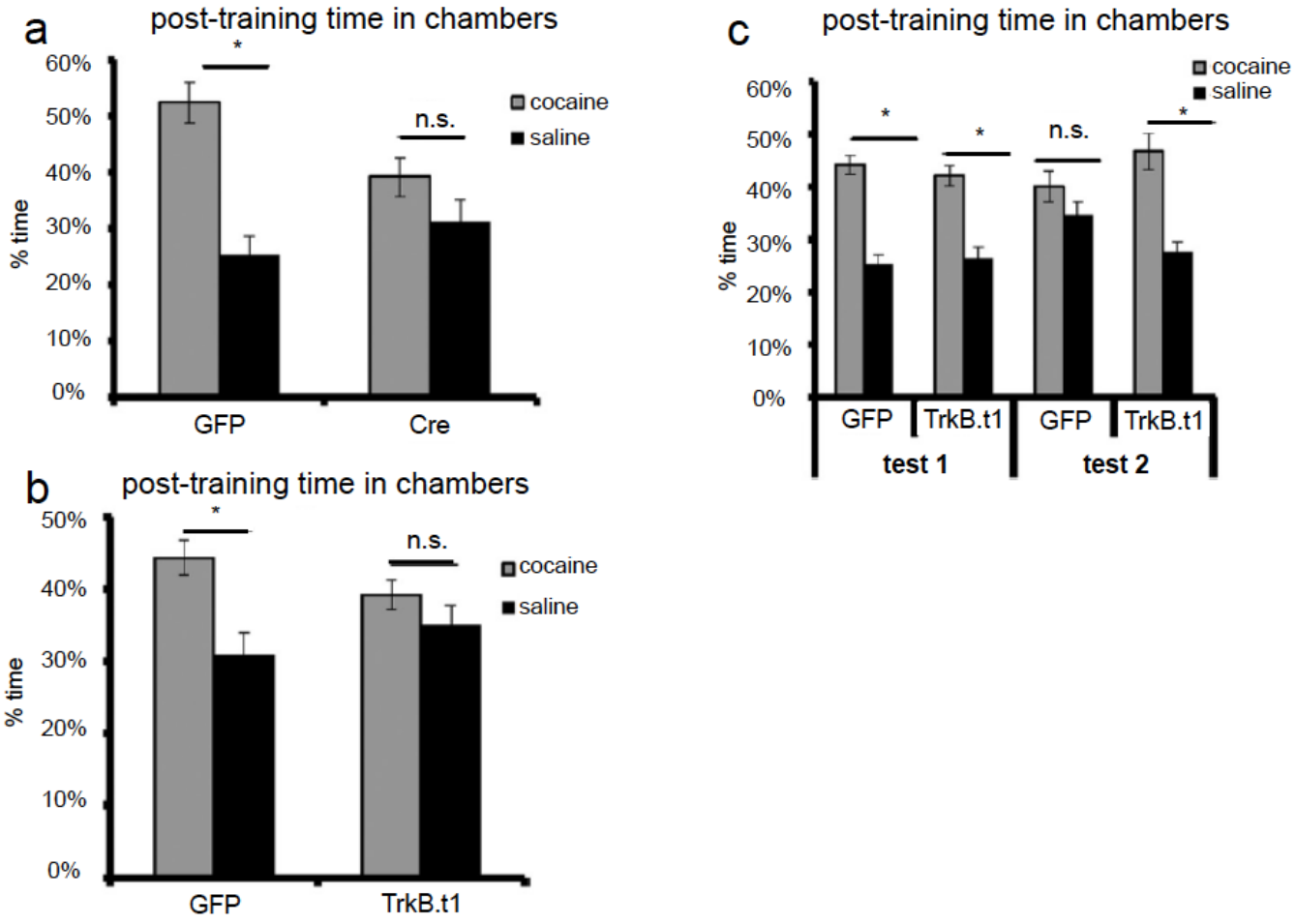
and control LV-GFP mice displayed similar levels of baseline anxiety-like behavior in the Elevated Plus Maze, and did not vary in percent time spent in the open arms or the percentage of entries into open arms. (d) Both LV-Cre and control LV-GFP mice displayed similar levels of motor activity as assessed by total ambulatory distance during EPM testing. (e) Diagrams from Paxinos and Franklin (2001) with red outlines indicating the hippocampal and amygdala regions of interest used for quantitative analyses of in situ hybridization studies. Symbols represent mean $\pm$ SEM. *$p<0.05$.

Figure 2-3: Bdnf *knockdown in the BLA disrupts FPS.*



(a) Percent FPS is graphed for the pre-training test (pretest) and subsequent post-training tests (post1 & post2). Mice infected with LV-GFP (GFP) demonstrate significant increases in their level of conditioned fear after training as measured with Percent FPS. In contrast, LV-Cre infected mice (Cre) showed no significant increase in Percent FPS across testing sessions. (b) Infusion maps, imposed on representative sections from Paxinos and Franklin (2001) Mouse Brain Atlas, for the maximal (left) and minimal (right) spread of LV-TrkB.t1 viral infusions. The actual infusions were bilateral, with no difference in left versus right infusion volumes. Bars represent means and SEMs, *$p<0.05$.

Figure 2-4: Bdnf *knockdown in the amygdala and impairment of TrkB prevent development of preference for a chamber previously paired with cocaine.*



GFP-infected mice spent significantly more time in the cocaine-paired chamber during post-testing, whereas Cre-infected mice (a) and TrkB.t1-infected mice (b) did not. (c) Once mice with TrkB.t1 infection of the BLA had formed a preference for the cocaine-paired chamber, they showed deficits in extinction, maintaining a preference after control mice had extinguished. Symbols and bars represent means and SEMs, *$p<0.05$.

Chapter 3:

Connections of the mouse orbitofrontal cortex and regulation of action selection by BDNF-TrkB

3.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter describes anatomical and functional connectivity between the OFC, striatum, and amygdala, and demonstrates the necessity of plasticity within the OFC and connectivity between the OFC and the amygdala for goal-directed decision-making. The dissertation author contributed to the paper by designing and running experiments, analyzing data, and was a main contributor to writing the manuscript, under the guidance of Dr. Gourley. Drs. Donald Rainnie and Kerry Ressler provided technological expertise and guidance, and contributed to editing the manuscript. Mr. John Yamin and Mr. Zach Liang assisted in running experiments. This chapter is reproduced from Zimmermann KS, Yamin JA, Rainnie DG, Ressler KJ, and Gourley SL (in revision) Connections of the mouse orbitofrontal cortex and regulation by BDNF-TrkB. *Biological Psychiatry.*

3.2 Abstract

Distinguishing between actions that are more, or less, likely to be rewarded is a critical aspect of goal-directed decision-making, however neuroanatomical and molecular mechanisms are not fully understood. We report that, as in other species, the mouse orbitofrontal cortex (OFC) projects to the basolateral amygdala and striatum, and that bilateral *Bdnf* knockdown within the ventrolateral OFC (VLO) impairs decision-making based on reward likelihood. Unilateral *Bdnf* knockdown, accompanied by lesions of the contralateral amygdala, also impedes goal-directed response selection, implicating BDNF-expressing VLO projection neurons in selecting actions based on their consequences. Furthermore, the TrkB agonist 7,8-dihydroxyflavone rescues action selection and also increases VLO dendritic spine density. Rho-kinase inhibition *also* rescues action selection strategies, directly linking neural remodeling with

outcome-based decision-making. Finally, DREADD-mediated VLO silencing weakens new action-outcome learning. Together, these findings indicate that activity- and BDNF-dependent neuroplasticity within the VLO coordinate outcome-based decision-making through interactions with the downstream amygdala.

3.3 Introduction

The orbitofrontal cortex (OFC) is essential for encoding information about rewards and translating this information into behavioral response strategies. Accordingly, both rodents and non-human primates with lesions or inactivation of the OFC fail to modify reward-seeking behaviors when the reward loses value (*e.g.,* see Rhodes and Murray 2013; McDannald et al. 2014). Further, experiments using reversal learning paradigms implicate the OFC in value judgment (Padoa-Schioppa, 2011) and outcome expectancy (Schoenbaum et al., 2009). In other words, across species, the OFC is critical for acquiring information relevant to salient outcomes.

These findings raise the possibility that the OFC may guide decision-making strategies based not just on outcome *value* or reward-related cues, but also on other outcome-related information such as the likelihood that a given response will result in a desired outcome. In line with this perspective, recent reports indicate that OFC-striatal interactions are preferentially engaged during goal-directed, as opposed to habitual, decision-making (Gremel and Costa, 2013). Further, perturbations in OFC-striatal interactions – through lesion, inactivation, hyper-activation, or targeted gene knockdown – result in involuntary motor movements, as well as inflexible stimulus-response habits (Ahmari et al., 2013; Gourley et al., 2013c; Gremel and Costa, 2013).

In addition to the striatum, the OFC projects to the basolateral nucleus of the amygdala (BLA) (McDonald et al., 1996), which is also necessary for goal-directed decision-making – that is, selecting an action based on the value of an anticipated reward, or based on the likelihood that the action will be reinforced (Balleine et al., 2003). From a circuit-level perspective, most reports have focused on BLA interactions with the nucleus accumbens and dorsal striatum (Wang et al., 2005; Shiflett and Balleine, 2010; Corbit et al., 2013), meaning top-down cortical regulation of BLA-dependent decision-making is uncharacterized in this context. Further, these and related studies have largely used lesion approaches in rats, leaving molecular mechanisms unclear. Finally, most studies of the BLA are restricted to outcome devaluation procedures, which assess decision-making based on the *value* of a goal, rather than the predictive relationship between a response and the goal.

In rats, as in primates, OFC-BLA projections are robust and organized topographically in the brain (McDonald, 1991; McDonald et al., 1996); however, this connectivity has not been as thoroughly characterized in the mouse – a widely used model organism in neurobiology. Therefore, in the present studies, we first verified that mouse OFC-amygdala projection patterns are homologous to those of rats and primates. Then, we used *in vivo* viral-mediated gene transfer to inactivate ventrolateral OFC (VLO) *Brain-derived neurotrophic factor* (*Bdnf),* and to silence the VLO using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), to test a model in which the VLO coordinates goal-directed action selection. We also used asymmetric infusion techniques to establish the functional necessity of VLO-BLA connectivity in selecting actions based on their consequences. We then attempted to augment goal-directed action selection using the TrkB agonist 7,8-dihydroxyflavone (7,8-DHF). Based on our evidence that 7,8-DHF induces dendritic spine proliferation, we last capitalized on the availability of a

blood brain barrier-penetrant Rho-kinase inhibitor to rescue outcome-based decision-making following *Bdnf* knockdown. Together, our findings indicate that VLO *Bdnf* systems organize goal-directed decision-making via interaction with the downstream BLA.

3.4 Materials and Methods

3.4.1 Subjects

Male C57BL/6 mice (Jackson Labs) were used except when knocking down *Bdnf* or enumerating dendritic spines. For knockdown, transgenic mice homozygous for a floxed allele (exon 5) encoding the *Bdnf* gene (Rios et al., 2001) were bred in-house on a BALB/c background. Dendritic spine analyses were conducted using transgenic mice expressing *thy1*-derived YFP that were fully back-crossed onto a B6 background (Feng et al., 2000). Mice were maintained on a 12-hour light cycle (0700 on), experimentally naïve, and ≥8 weeks old. Mice were provided food and water *ad libitum* except during instrumental conditioning when body weights were maintained at ~93% of baseline to motivate responding. Procedures were approved by the Emory University IACUC.

3.4.2 Surgery.

Mice were anaesthetized with ketamine/xylazine, ketamine/dormitor, or 1:1 2-methyl-2-butanol and tribromoethanol (Sigma) diluted 40-fold with saline in the case of lesions. With needles centered at bregma, sterotaxic coordinates were located on the leveled skull using a digitized stereotaxic frame (Stoelting). For anatomical tracing experiments, mice received 0.15 μl unilateral infusions of BDA 10,000 in either VLO or DLO/AI. Coordinates from bregma, based on the mouse brain atlas of Paxinos and Franklin (2001), were as follows: AP+2.22 to

+2.8, ML±1.2 to ±2.2, DV-2.8 to -3.0. The microsyringe remained in place for 10 min following infusions. One week following surgery, mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were collected and BDA expression was analyzed as described below under "Histology."

For viral vector and lesion surgeries, lentiviral vectors expressing GFP or Cre Recombinase were generated by the Emory University Viral Vector Core. AAV5-CaMKII-HA-hM$_4$D(Gi)-IRES-mCitrine or AAV5-CaMKII-GFP were purchased from the UNC Viral Vector Core. Infusion coordinates for the VLO were AP+2.6, DV-2.8, ML±1.2 (Bissonette et al., 2008; Gourley et al., 2010; Gourley et al., 2013b). Amygdala coordinates were AP-1.4, DV-4.9, ML±3.0. Viral vector volumes were 0.25 μl; 0.1 μl was used for NMDA (20 μg/μl in sterile saline; Sigma). Infusions were delivered over 5 minutes with needles left in place for 4 additional min. Mice were sutured and allowed to recover for at least 3 weeks before behavioral testing. After testing, mice were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde, then brains were processed as described under "Histology."

Vectors were infused bilaterally, or in the case of "disconnection" experiments, lenti-Cre was infused *unilaterally* in the VLO and NMDA was infused either ipsilaterally or contralaterally in the amygdala. Additional mice were infused ipsilaterally or contralaterally with lenti-GFP and saline, respectively. Throughout, no differences were observed between these two control groups, which were combined for statistical and graphical purposes.

3.4.3 Instrumental conditioning.

Mice were trained to nose poke for food reinforcement (20 mg grain-based pellets; Bioserv) using illuminated Med-Associates conditioning chambers. Training was initiated with a

continuous reinforcement schedule; 30 pellets were available for responding on each of 2 distinct nose poke recesses located on opposite sides of a single wall within the chambers, resulting in 60 pellets/session. Sessions ended when all 60 pellets were delivered or at 135 min. After 5 sessions, mice were shifted to a random interval (RI) 30 second schedule of reinforcement for 2 sessions or as indicated; again, 30 pellets were available for responding on each of 2 apertures. At this point, sensitivity to instrumental contingency degradation was tested, or in the case of extended training, mice were trained for an additional 6 RI30-second sessions and then 7 RI60-second sessions to promote the formation of stimulus-response habits (Dickinson et al., 1983). Response acquisition curves represent total responses/minute.

Instrumental contingency degradation was accomplished during two conditioning sessions, the order of which was counter-balanced (Gourley et al., 2013b; Gourley et al., 2013a; Swanson et al., 2013; Hinton et al., 2014): In the 'non-degraded session,' one nose poke aperture was occluded, and responding on the other aperture was reinforced using a variable ratio 2 schedule of reinforcement for 25 minutes. In the 'degraded session', the opposite aperture was occluded, and reinforcers were delivered into the magazine for 25 minutes at a rate matched to each animal's reinforcement rate the previous day. Responding produced no programmed consequences. Thus, one response became significantly more predictive of reinforcement than the other (see (Hinton et al., 2014)) for response-pellet delivery probability distributions). Both apertures were available during a subsequent 10-min probe test, during which responding was non-reinforced. In the "disconnection" experiment, this 3-day process was repeated. Response rates were compared by 2-factor (aperture x group) analysis of variance (ANOVA).

3.4.4 Extinction conditioning.

After testing as above, *thy1*-YFP mice in one experiment were placed in the conditioning chambers for an additional 15 minute/day for 7 days. Responding was not reinforced, and mice were injected with saline or 7,8-DHF immediately after each session. This protocol is sensitive to between-group differences in appetitive response extinction (Gourley et al., 2009). Response rates on both apertures were compared by ANOVA with repeated measures.

3.4.5 Drug treatment.

7,8-DHF (Sigma; 5 mg/kg, i.p., in 17% DMSO + PBS), fasudil (LKT Laboratories; 10 mg/kg, i.p., in PBS), CNO (Sigma; 1 mg/kg, i.p., in 2% DMSO + PBS), or the corresponding vehicle was administered immediately following action-outcome contingency degradation or extinction training, as indicated. This experimental design allowed us to target the consolidation, rather than acquisition or expression, of conditioning.

3.4.6 Dendritic spine imaging and enumeration.

Dendritic spine imaging was accomplished as described (Gourley et al., 2012b; Gourley et al., 2013a). Fresh YFP-expressing brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 40 μm-thick sections on a microtome held at -15°C. Unobstructed dendritic segments running parallel to the surface of the section were imaged on a spinning disk confocal (VisiTech International) on a Leica microscope. Z-stacks were collected with a 100x 1.4NA objective using a 0.1 μm step size, sampling above and below the dendrite. After imaging, we confirmed at 10x that the image was collected from the VLO.

Collapsed z-stacks were analyzed using NIH ImageJ: Each protrusion ≤4 μm was considered a spine (Peters and Kaiserman-Abramof, 1970). Individual planes were evaluated to detect protrusions perpendicular to the z-stack. Bifurcated spines were considered singular units. To generate density values, spine number for each segment was normalized to the length of the segment. Four-6 independent segments from secondary and tertiary dendritic branches within 50-150 μm of the soma were collected. Each animal contributed a single density value (its average) to statistical analyses. Due to the relatively stellate appearance of VLO neurons, apical *vs*. basal branches were not distinguished (Liston et al., 2006; Kolb et al., 2008). A single blinded rater scored all spines.

3.4.7 Histology.

Brains were sectioned into 55 μm-thick sections on a microtome held at -15°C. For BDA tracing studies, BDA signal was amplified with a standard Vectastain Elite ABC kit and revealed by nickel-enhanced-diaminobenzidine staining. Sections were then mounted and lightly counterstained with Cresyl Violet before being coverslipped. BDA signal was examined in both brightfield and darkfield. Maximum diffusion around the infusion site was mapped, and labeling patterns of axon terminals from each infusion site were transposed onto representative coronal sections from *The Mouse Brain in Stereotaxic Coordinates, Second Edition* (Paxinos and Franklin, 2001). Labeling from 2-3 animals was analyzed for each site.

In the case of lentiviral vector delivery, every third section was imaged for GFP, or immunostained for Cre as described (DePoy et al., 2013). For DREADD experiments, GFP or mCitrine was visualized as appropriate.

To confirm lesion sites, every third section was immunostained for Glial Fibrillary Acidic Protein (GFAP) (Dakocytomation; Ms; 1:1000) as described (Gourley et al., 2010).

3.4.8 BDNF quantification.

In one cohort of mice with bilateral VLO *Bdnf* knockdown (from fig.3-5), mice were rapidly decapitated, and brains were immediately frozen on dry ice, rather than perfused, for BDNF quantification by enzyme-linked immunosorbent assay (ELISA). Frozen tissue was sectioned into 1 mm-thick coronal sections, and the amygdala was extracted with bilateral tissue punches (1 mm core) and homogenized in lysis buffer [200 μl: 137 mM NaCl, 20 mM tris-Hcl (pH=8), 1% igepal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 1 and 2 (Sigma)] by sonication.

BDNF quantification was accomplished using a 2-site BDNF ELISA kit in accordance with the manufacturer's instructions (Promega), except tissue was diluted 1:1, and the extraction procedure was excluded. BDNF concentrations were normalized to the total protein content in each sample; covariance with behavioral measures was tested using a linear regression analysis.

3.4.9 Statistical analyses.

Two-tailed parametric statistical analyses with $\alpha \leq 0.05$ were performed using SigmaStat v.3.1 or SPSS. Tukey's post-hoc tests were utilized in the event of significant interaction effects; significant posthoc comparisons are indicated graphically. In the case of values lying >2 standard deviations outside of the mean, values were excluded.

3.5 Results

3.5.1 OFC projections to the dorsal striatum, BLA, and perirhinal cortex are topographically organized in the mouse.

The projections between the OFC, dorsal striatum, and amygdala have been extensively described in primates (Barbas, 2000; Groenewegen and Uylings, 2000; Barbas, 2007), and in rats (McDonald, 1991; McDonald et al., 1996; McDonald, 1998; Schilman et al., 2008). Limited literature exists describing this connectivity in mice. We thus initiated these studies by infusing the anterograde tracer biotinylated dextran amine (BDA)-10,000 into the VLO or DLO/AI. We then imaged BDA in the striatum, amygdala, and perirhinal cortex.

Following BDA infusion into the VLO (fig.3-1a), innervation of both the dorsal striatum and amygdala by the VLO was overwhelmingly unilateral. The central aspect of the dorsal striatum received heavy innervation broadly along the rostrocaudal axis (fig.3-1b). Light labeling was also present in the ventral striatum. Fibers entered the rostral striatum through the genu of the corpus callosum (gcc) and the external capsule, then formed multiple fiber bundles that coursed through the dorsomedial terminal fields along the rostrocaudal axis. Much of the innervation of the striatum originated from terminals emerging from these fiber bundles (fig.3-2a,b). Overall, projection patterns closely resembled those reported in rats (McDonald, 1991; Schilman et al., 2008).

Within the amygdala, VLO-originating fibers largely spared the dorsal portion of the lateral amygdala and instead targeted the BLA (fig.3-1c,d). In rostral sections, innervation was widely distributed, but in more caudal sections, labeling became laterally oriented along the external capsule. Light innervation of the medial intercalated masses was noted, but the central nucleus was relatively devoid of labeled terminals. Fibers originating in the VLO appeared to reach posterior targets primarily through the external and internal capsules. Although BDA

10,000 acts primarily as an anterograde tracer, retrograde labeling was evident to varying degrees in the BLA of mice that received VLO infusions (fig.3-2c). These findings suggest bidirectional connectivity, consistent with prior reports (Ghashghaei and Barbas, 2002; Matyas et al., 2014). Projections from the VLO to the amygdala were overwhelmingly ipsilateral — no labeled terminals were found contralateral to the infusion site.

BDA infusions targeting the DLO/AI (fig.3-3a) resulted in strong labeling in both hemispheres of the dorsal and ventral striatum (fig.3-3b), though innervation was heaviest in the hemisphere ipsilateral to the infusion site (fig.3-4a). Unlike the VLO, the DLO/AI most heavily innervated the lateral and ventral striatum. Labeling was heavier in more posterior striatal sections, culminating in a massive innervation of the posterior caudate (fig.3-4b). Again, the projection patterns strongly resembled those reported in rats (McDonald, 1991; Schilman et al., 2008). Similar to the VLO, fibers originating from the DLO/AI reached the rostral striatum through the gcc and the external capsule and were organized into fiber bundles. Labeled bundles were present only in the hemisphere ipsilateral to the infusion site, and were generally located dorsally to the primary site of innervation.

Projections from the DLO/AI to the amygdala were almost exclusively unilateral. Innervation was again topographically organized (fig.3-3c,d); the heaviest labeling was identified in the rostral BLA and — similar to the VLO — terminals were densest along the lateral wall of the BLA (fig.3-3d;3-4c). As reported by McDonald et al. (1996), mid-amygdaloid labeling was primarily evident in the lateral aspects of the basal nucleus, along with the ventral portion of the lateral amygdala. The majority of terminals in posterior sections were located in the ventrolateral field of the basal nucleus (fig.3-3d;3-4c). Additionally, we identified discrete but substantive innervation of the ventral basolateral complex in mid- and caudal-amygdaloid sections. We

found little evidence of innervation of the central nucleus or the intercalated masses by the DLO/AI. Fibers in the amygdala appeared to arrive at the target via the external capsule.

Additionally of note was the presence of terminals and fibers of passage in the perirhinal cortex (PRh) originating from the DLO/AI, suggesting a DLO/AI-perirhinal-hippocampus pathway in mice similar to that found in macaques (Van Hoesen et al., 1975; Insausti et al., 1987; Suzuki and Amaral, 1990). Infusions into the DLO/AI revealed innervation along the entire rostrocaudal spread of the AI cortex, which continued well beyond the AI-PRh transition (fig.3-4c).

3.5.2 VLO-amygdala interactions coordinate outcome-based decision-making.

To summarize, the mouse VLO projects to the dorsomedial striatum and BLA, two regions associated with goal-directed action selection (Corbit et al., 2002; Balleine et al., 2003; Yin et al., 2008; Lovinger, 2010). We thus hypothesized that the VLO might *itself* regulate decision-making based on the predictive relationship between an action and an outcome. To test this hypothesis, we used a task in which mice were trained to generate two operant responses, then the likelihood that one response would be reinforced was reduced (action-outcome contingency degradation). Meanwhile, the other response remained reinforced. Thus, one response became significantly more predictive of reinforcer delivery than the other (Hinton et al., 2014), and response strategies — "goal-directed" *vs.* "habitual" — were reflected during a subsequent probe test when both apertures were simultaneously available (fig.3-5a). Throughout, response acquisition curves reflect total responding on both apertures, and response rates during the probe tests are shown.

We first selectively knocked down the neurotrophin *Bdnf* in the VLO using Cre Recombinase-expressing viral vectors and 'floxed' *Bdnf* mice. Infusion sites throughout were largely restricted to the VLO with minimal diffusion into the medial OFC and DLO/AI (fig.3-5b). During response acquisition, response rates in the knockdown group lagged, particularly in later sessions when the reinforcement schedule escalated from a fixed ratio to a random interval [interaction $F_{(6,66)}$=3.8, p=0.006] (fig.3-5c). This profile has been associated with an impairment in decision-making based on action-outcome contingencies (Corbit and Balleine, 2003), and indeed, mice with bilateral VLO *Bdnf* knockdown subsequently failed to differentiate between responses that were more, or less, likely to be reinforced, instead relying on familiar habit-based strategies and responding equivalently on both apertures following instrumental contingency degradation [interaction $F_{(1,22)}$=9, p=0.007] (fig.3-5d).

Selective prefrontal cortical *Bdnf* knockdown decreases BDNF expression in both the dorsal striatum and amygdala (Gourley et al., 2009; Gourley et al., 2013c), consistent with evidence that cortical pyramidal neurons provide a substantial source of BDNF to downstream targets via axonal transport (Altar et al., 1997; Conner et al., 1997). For this reason, we measured BDNF protein in the amygdala in mice with selective bilateral VLO knockdown. Amygdala BDNF levels correlated with decision-making strategies, in that higher amygdala BDNF levels were associated with higher rates of responding on the non-degraded aperture (r=0.53, p=0.05), while "low" BDNF was associated with habits (fig.3-5e). This pattern is suggestive of a model in which BDNF-expressing amygdala-targeted VLO projection neurons regulate action selection.

To test this model, we next modified classical disconnection procedures in which contralateral lesions would be placed in the VLO and BLA. We instead knocked down *Bdnf*

unilaterally in the VLO and infused NMDA in either the ipsilateral or contralateral BLA to generate lesions. Lesions were largely contained within the basal and central nuclei (fig.3-6a).

Following these manipulations, all mice acquired the instrumental responses, with no differences between groups (interaction $F<1$) (fig.3-6b). Thus, the response acquisition deficits following bilateral VLO *Bdnf* knockdown cannot obviously be attributed to the effects of *Bdnf* knockdown on interactions with the BLA. By contrast, mice with contralateral infusions responded equivalently on the degraded and non-degraded apertures following instrumental contingency degradation, habitually [interaction $F_{(2,30)}=4.9$, $p<0.05$] (fig.3-6c). Both GFP-expressing control mice and mice with ipsilateral infusions, leaving one cortico-amygdalar circuit intact, differentiated between the two responses in a goal-directed fashion.

With additional contingency degradation training, mice with contralateral infusions ultimately differentiated between the responses (fig.3-6c). These data suggest that inhibiting BDNF-dependent VLO-amygdala interactions delays, but *does not fully block*, expression of action-outcome decision-making.

3.5.3 Augmenting TrkB activity increases sensitivity to action-outcome associations.

Together, our results indicate that VLO-derived BDNF is necessary for goal-directed action selection. Therefore, we next attempted to *rescue* goal-directed action selection by pharmacologically stimulating the high-affinity BDNF receptor TrkB using the small-molecule agonist 7,8-DHF (Jang et al., 2010). First, we extensively trained intact mice such that they would be expected to develop stimulus-response habits by virtue of prolonged task experience. There were no differences in response acquisition between mice ultimately designated to vehicle or 7,8-DHF groups ($F<1$) (fig.3-7a). When we degraded one of the two action-outcome

contingencies and injected mice immediately following this training session (during the presumptive consolidation of new learning), vehicle-treated mice failed to differentiate between the responses the following day, instead relying on familiar habit-based strategies as expected. By contrast, mice treated with 7,8-DHF generated the response most likely to be reinforced nearly twice as often as the 'degraded' response [interaction $F_{(1,12)}$=6.2, p=0.03] (fig.3-7b). Thus, 7,8-DHF blocked habits by enhancing outcome-based conditioning.

Next, we trained separate mice to nose poke using a continuous reinforcement schedule (fig.3-7c) and confirmed that systemic 7,8-DHF had no effects at a time point when typical mice would be expected to be "goal-directed." This is important because prelimbic prefrontal cortex-targeted BDNF microinfusions have been shown, under certain circumstances, to cause habit-like behavior (Graybeal et al., 2011; Gourley et al., 2012b). Nonetheless, a main effect of response indicated that sensitivity to instrumental contingency degradation was intact [$F_{(1,13)}$=71.2, p<0.001] (fig.3-7d).

It has long been appreciated that sensitivity to action-outcome contingency degradation and non-reinforcement (*i.e.*, extinction) are dissociable (Hammond, 1980), and while the OFC is involved in appetitive extinction conditioning, it is not a site of extinction consolidation *per se* (Panayi and Killcross, 2014). On the other hand, 7,8-DHF *enhances* the extinction of freezing after fear conditioning (Andero et al., 2011), raising the possibility that it may also act on the extinction of an appetitive response. Thus, we trained mice further until responding was robust (>4 responses/min), then withheld reinforcement entirely. Despite injections immediately following each extinction training session, 7,8-DHF did not impact response extinction (Fs<1) (fig.3-7e). Together, our results suggest that stimulating TrkB activity selectively enhances the ability of mice to select between actions based on their consequences, rather than inhibit actions

when no reinforcement is available. Following response extinction, we enumerated dendritic spines in the VLO. As has been reported in the hippocampus (Zhang et al., 2014), 7,8-DHF increased deep-layer dendritic spine density (fig.3-7f).

3.5.4 7,8-DHF and Rho-kinase inhibition correct response strategies following Bdnf silencing.

We next generated another group of mice with VLO-targeted *Bdnf* knockdown. We aimed to assess whether 7,8-DHF could correct response strategies in these mice. Based on evidence that 7,8-DHF increases VLO dendritic spine density, we additionally evaluated whether augmenting structural plasticity more directly could correct response strategies. This outcome would provide evidence that the structural effects of 7,8-DHF are associated with the regulation of outcome-based decision-making. As above, we administered 7,8-DHF or the Rho-kinase inhibitor HA-1077 (fasudil) immediately following contingency degradation training. Fasudil was selected because Rho-kinase inhibitors enhance activity-dependent dendritic spine plasticity (Murakoshi et al., 2011), and fasudil is brain-penetrant.

We designated treatment groups by matching mice based on response acquisition curves, and there were no differences between groups ("to be 7,8-DHF" *vs.* "to be saline" *vs.* "to be fasudil" $F<1$) (fig.3-7g). As expected, *Bdnf* knockdown reduced response rates during instrumental response acquisition (main effect of *Bdnf,* $p=0.04$). Subsequently, vehicle-treated mice with VLO-targeted *Bdnf* knockdown failed to differentiate between the responses that were more, or less, likely to be reinforced as expected; by contrast, mice with *Bdnf* knockdown followed by systemic 7,8-DHF or fasudil intervention showed evidence of goal-directed decision-making, preferentially engaging the non-degraded response [*Bdnf* x 7,8-DHF interaction $F_{(1,37)}=4$, $p=0.05$; effect of fasudil $t_5=2.6$, $p=0.047$] (fig.3-7h).

As further evidence that 7,8-DHF and fasudil rescued response selection strategies, we compared the percentage of total responses directed towards the non-degraded nose poke aperture, *i.e.*, the response most likely to be reinforced. In control mice, >60% of responses were directed toward the non-degraded aperture; this preference dropped to chance levels in *Bdnf* knockdown mice, but was fully rescued with 7,8-DHF and fasudil intervention [$F_{(4,39)}$=6.8, p<0.001; all groups compared to Cre-only, $p\leq$0.04] (fig.3-7i).

3.5.5 Gi-DREADD-mediated VLO silencing impairs goal-directed action selection.

Our dendritic spine counts suggest that 7,8-DHF augments local VLO plasticity, leading to the prediction that inhibition of intracellular second-messenger systems in the VLO would impair goal-directed action selection by disrupting new learning. To test this model, we infused into the VLO mCitrine-tagged CaMKII-derived Gi-DREADD (fig.3-8a), a designer Gi-protein-coupled receptor that is inactive until administration of clozapine-n-oxide (CNO), its synthetic ligand. Control mice expressed GFP. Groups did not differ during instrumental response acquisition when CNO was not present (fig.3-8b). We systemically administered CNO to both groups immediately following contingency degradation, then tested response selection the following day when mice were drug-free. Response rates are shown in 5-min bins (fig.3-8c). Control mice consistently directed responding towards the aperture more closely associated with reinforcement delivery [main effect $F_{(1,6)}$=7.1, p=0.04] (fig.3-8c, left). By contrast, mice expressing the Gi-DREADD initially directed responding towards the aperture most likely to be reinforced, but this effect decayed, and responding was ultimately non-selective [interaction $F_{(1,5)}$=24.6, p=0.004] (fig.3-8c, right). An overall interaction further indicated that silencing the

OFC weakened the consolidation of new information regarding action-outcome relationships [interaction $F_{(1,11)}$=5.1, p<0.05] (fig.3-8d).

3.6 Discussion

Considerable evidence indicates that the OFC encodes salient information regarding desired outcomes, such as external cues signaling reinforcement, as well as the value of rewards (McDannald et al., 2014). The OFC may also guide outcome-based decision-making based on other reinforcement-related information such as the likelihood that a given behavior will be reinforced. This is consistent with evidence implicating the OFC in drug seeking in addiction (Lucantonio et al., 2012) and unremitting anhedonic-like behavior in models of depression (Gourley et al., 2013a), but few investigations into OFC function have focused on action-outcome associative learning and memory. We hypothesized that action selection based on the likelihood of reward recruits an OFC-amygdala neurocircuit, which has been characterized in the rat, but less defined in the mouse. We addressed these gaps in current knowledge by first showing that two important subregions of the OFC — the VLO and DLO/AI — project to the amygdala in mice in patterns similar to those reported in rats (McDonald, 1991; McDonald et al., 1996). Next we used a combination of site-selective gene silencing and pharmacological interventions to demonstrate that: *1)* VLO-derived BDNF is required for goal-directed decision-making; *2)* obstructing BDNF-dependent functional connectivity between the VLO and amygdala impairs goal-directed decision-making; *3)* habitual response strategies induced by either extended training or selective VLO *Bdnf* knockdown can be reversed by the TrkB agonist, 7,8-DHF, or the Rho-kinase inhibitor, fasudil; and *4)* DREADD-mediated inhibition of VLO

excitability disrupts the consolidation of new information regarding the predictive relationship between actions and their outcomes, weakening goal-directed response strategies.

We have previously reported that VLO-striatal interactions also regulate goal-directed action selection, and that knocking down *Bdnf* in the VLO reduces amygdalo-striatal BDNF protein levels (Gourley et al., 2013c). Here, we expand on these findings by describing the anatomical connectivity between the OFC and striatum in the mouse and further exploring its anatomical and functional interactions with the amygdala. Our anatomical tracing experiments indicate that both subregions of the OFC examined — the VLO and DLO/AI — project to the BLA, an amygdalar subdivision critically involved in associative learning and memory (Davis, 1992; Fanselow and LeDoux, 1999) and goal-directed decision-making (Balleine et al., 2003). These patterns are highly consistent with those reported in monkeys (Barbas, 2000; Groenewegen and Uylings, 2000; Barbas, 2007) and rats (McDonald, 1991; McDonald et al., 1996). Conservation across species suggests that these networks are essential for evolutionarily-conserved behaviors, such as learning that specific actions can produce desired outcomes.

These projection patterns also highlight that the VLO is uniquely positioned to provide top-down regulation of actions and habits through interactions with the BLA. This may occur via local plasticity within the OFC that in turn coordinates differential excitatory outputs. In line with this perspective, we report that VLO-selective knockdown of *Bdnf,* which regulates activity-dependent neuroplasticity, impairs the ability of mice to select between response strategies that are more, or less, likely to be reinforced. Additionally, the OFC may affect plasticity in the downstream BLA via axonal transport of small peptides such as BDNF. Consistent with this perspective, we also report that BDNF expression *in the amygdala* predicts action selection strategies following VLO-selective *Bdnf* knockdown.

Importantly, we find that VLO-BLA projections are both topographically-organized and ipsilateral, as in other species. We capitalized on this segregated neuroanatomy by knocking down VLO *Bdnf* unilaterally and ablating the contralateral amygdala, leaving the infected VLO to project to the one remaining healthy amygdala. The benefit of this "disconnection" approach, when combined with the appropriate symmetric infusion control group, is that it allows for the assessment of the impact of BDNF-dependent VLO-BLA *interactions* on instrumental decision-making. As expected, contralateral infusions resulted in reflexive habits, recapitulating the effects of bilateral VLO *Bdnf* knockdown. Importantly, goal-directed responding was spared in mice with ipsilateral infusions, in which one OFC-amygdala circuit remained intact, further indicating that BDNF-mediated connectivity between these two structures is fundamental to optimal action selection.

TrkB regulates the consolidation of action-outcome conditioning

Site-selective *Bdnf* silencing impaired goal-directed action selection, raising the possibility that *amplification* of TrkB activity could conversely *rescue*, or *enhance*, action-outcome conditioning. We first extensively trained intact mice such that they would be expected to develop stimulus-response habits by virtue of prolonged task experience (Dickinson et al., 1983). As expected, vehicle-treated mice were insensitive to degradation of the instrumental contingency, a classical indicator of habit formation (Balleine and O'Doherty, 2010). By contrast, the novel TrkB agonist 7,8-DHF rescued goal-directed action selection strategies. These and other reports using similar approaches indicate that a latent "goal-directed" system can be accessed even once habits have developed (Kimchi et al., 2009; Gourley et al., 2013b; Swanson et al., 2013), and our findings indicate that this process is TrkB-sensitive. We also treated mice

with 7,8-DHF following VLO-targeted *Bdnf* knockdown. Amplification of TrkB binding again rescued action selection. This finding importantly suggests that BDNF organizes action selection through its high-affinity receptor TrkB, as opposed to pro-BDNF binding to the p75 receptor.

In these experiments, we administered 7,8-DHF immediately following action-outcome contingency degradation, rather than at the probe test when mice must choose between responses that are more, or less, likely to be reinforced. This experimental design was motivated by evidence that temporary inactivation of the BLA during outcome devaluation training occludes goal-directed response selection during a subsequent probe test, while inactivation *during* the probe test has no effects (Wellman et al., 2005; West et al., 2012; Parkes and Balleine, 2013). Thus, the BLA is essential for learning about, but not necessarily expressing, goal-directed decision-making strategies. Injections immediately following the training sessions additionally allowed us to avoid drug effects on the *acquisition* of instrumental contingency degradation training and instead target the consolidation phase of new learning.

Considerable attention has been given to the functional significance of projections *from* the BLA *to* the OFC (Schoenbaum et al., 2003; Holland and Gallagher, 2004). While we have instead focused on OFC projections *to* the BLA, it seems probable that bidirectional interactions regulate BDNF-dependent action selection. For example, 7,8-DHF increased dendritic spine density in deep-layer VLO. This spine population is targeted by BLA projections (Ghashghaei and Barbas, 2002), so it is conceivable that 7,8-DHF corrected decision-making strategies in a *direct* manner by restoring VLO TrkB binding, and in an *indirect* manner by structurally remodeling these neurons to support greater synaptic connectivity and increased sensitivity to BLA inputs. Supporting this "indirect" model, Rho-kinase inhibition also corrected decision-making strategies in *Bdnf*-deficient mice. Rho-kinase provides a contractile force on the actin

cytoskeleton, and inhibitors *augment* activity-dependent structural plasticity (Murakoshi et al., 2011) and elaborate neuron structure (Couch et al., 2010). A final consideration is that 7,8-DHF may have acted by additionally increasing TrkB binding *in the amygdala* and augmenting long-term potentiation in this region (Li et al., 2011a).

Based on the association between structural plasticity in the VLO and regulation of action selection strategies, as well as evidence that BDNF release from axons of pyramidal neurons is activity-dependent (Balkowiec and Katz, 2002; Gartner and Staiger, 2002; Jia et al., 2010), we also used a Gi-coupled DREADD to selectively silence excitatory VLO neurons. When the synthetic ligand CNO is administered, an inhibitory Gi pathway is activated, reducing the likelihood that Gi-DREADD-expressing neurons will generate activity-dependent action potentials (Dong et al., 2010). We hypothesized that impeding activity-dependent excitatory transmission would disrupt the consolidation processes associated with developing goal-directed action selection strategies, rendering the memory of contingency degradation training inherently labile. Indeed, stimulating the Gi-DREADD following action-outcome contingency degradation prevented stable consolidation of new action-outcome associations.

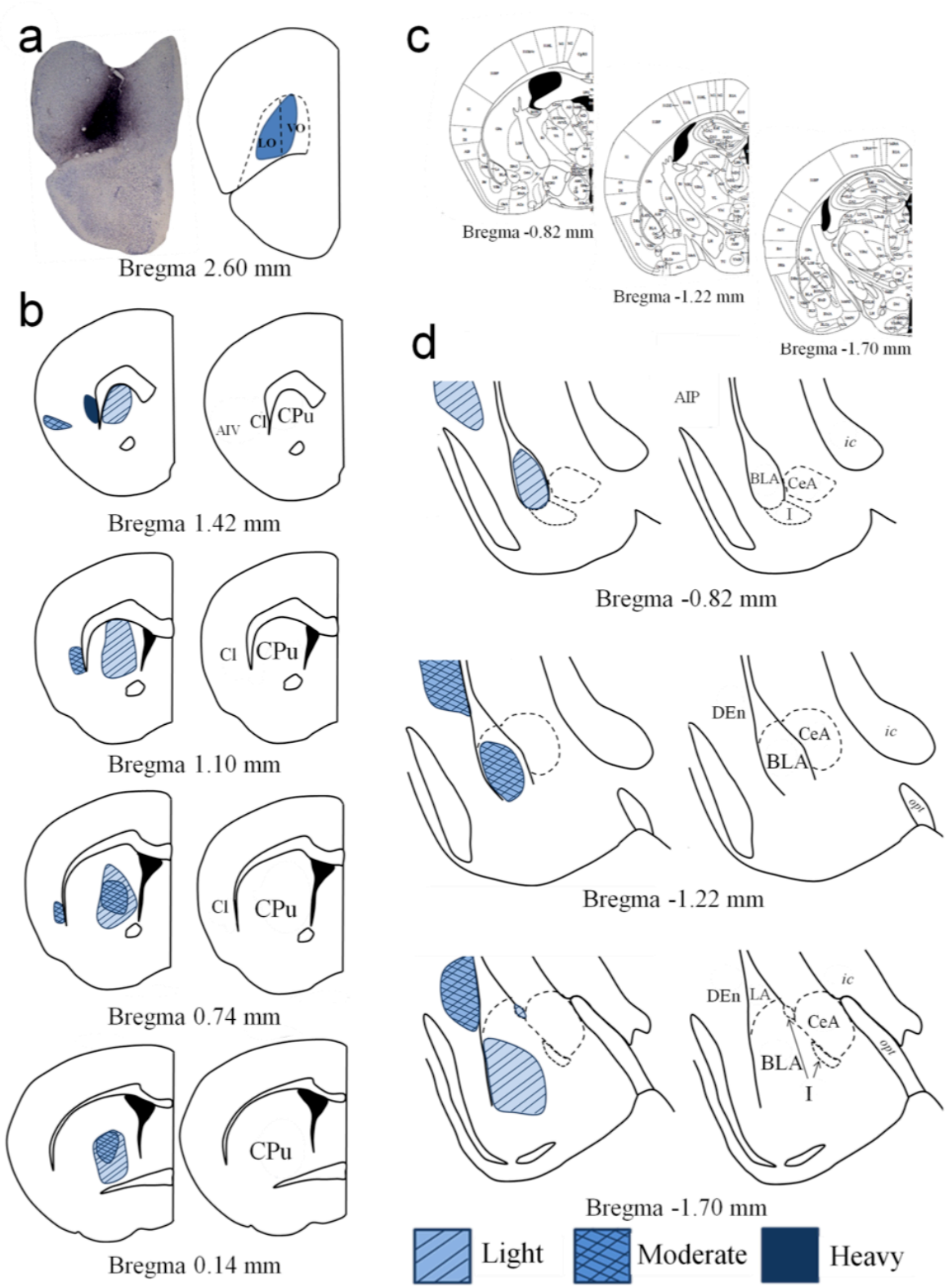
DREADD-mediated silencing of the mediodorsal thalamus (MD) also occludes sensitivity to action-outcome contingency degradation. The MD shares rich reciprocal innervation with both the OFC and the BLA in rats and mice (Groenewegen, 1988; Matyas et al., 2014), a pattern we also noted in the course of our tracing studies (not shown). In rhesus macaques, the BLA projects heavily to an area of MD that in turn projects robustly to the OFC (Barbas et al., 2011) — notably, the same group found that terminals originating from the thalamus and those arising directly from the BLA developed distinct innervation patterns in the OFC (Timbie and Barbas, 2014), suggesting the possibility of both direct and indirect pathways

between the OFC and subcortical structures with unique functions. These pathways may act synergistically to relay and modify information in situations that require updating of previously-learned action-outcome contingencies.

Conclusions

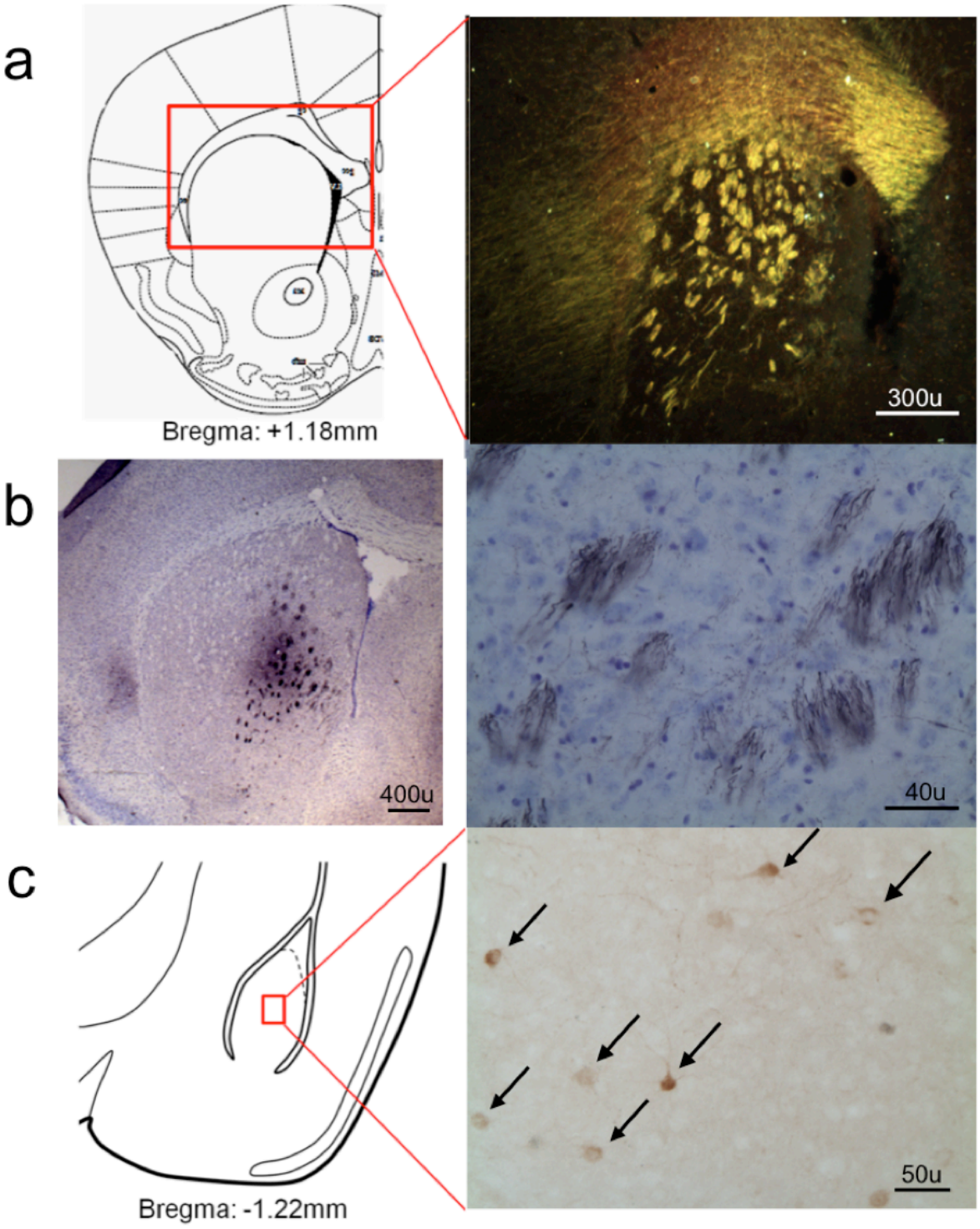
Our findings implicate for the first time a BDNF-sensitive VLO-amygdala neurocircuit in the bidirectional regulation of actions and habits. Why is this relevant to human health? First, *BDNF* status and OFC structure and function are linked. For example, prenatal nicotine exposure and familial alcoholism interact with *BDNF* met status to impact OFC thickness (Hill et al., 2009; Lotfipour et al., 2009). In animal models, cocaine seeking is associated with elevated OFC *Bdnf* (Hearing et al., 2008), and diminished OFC *Bdnf* increases sensitivity to cocaine-associated conditioned stimuli (Gourley et al., 2013c). Given our current findings, it is tempting to speculate that drug-related OFC *Bdnf* overexpression drives goal-oriented drug seeking, while the atrophy of OFC neurotrophin systems — caused by stress hormone exposure, for example (Gourley et al., 2009) — can drive habitual drug seeking. Second, identifying experimental techniques to reverse habits has proven quite challenging in the field. Thus, an additional critical finding here is that TrkB-targeted drugs may serve as promising adjuncts to behavioral therapies aimed at suppressing or reversing habitual, maladaptive thought or behavioral patterns.

Figure 3-1: *The VLO innervates the dorsal and central striatum and projects to the BLA and ITCs of the amygdala.*



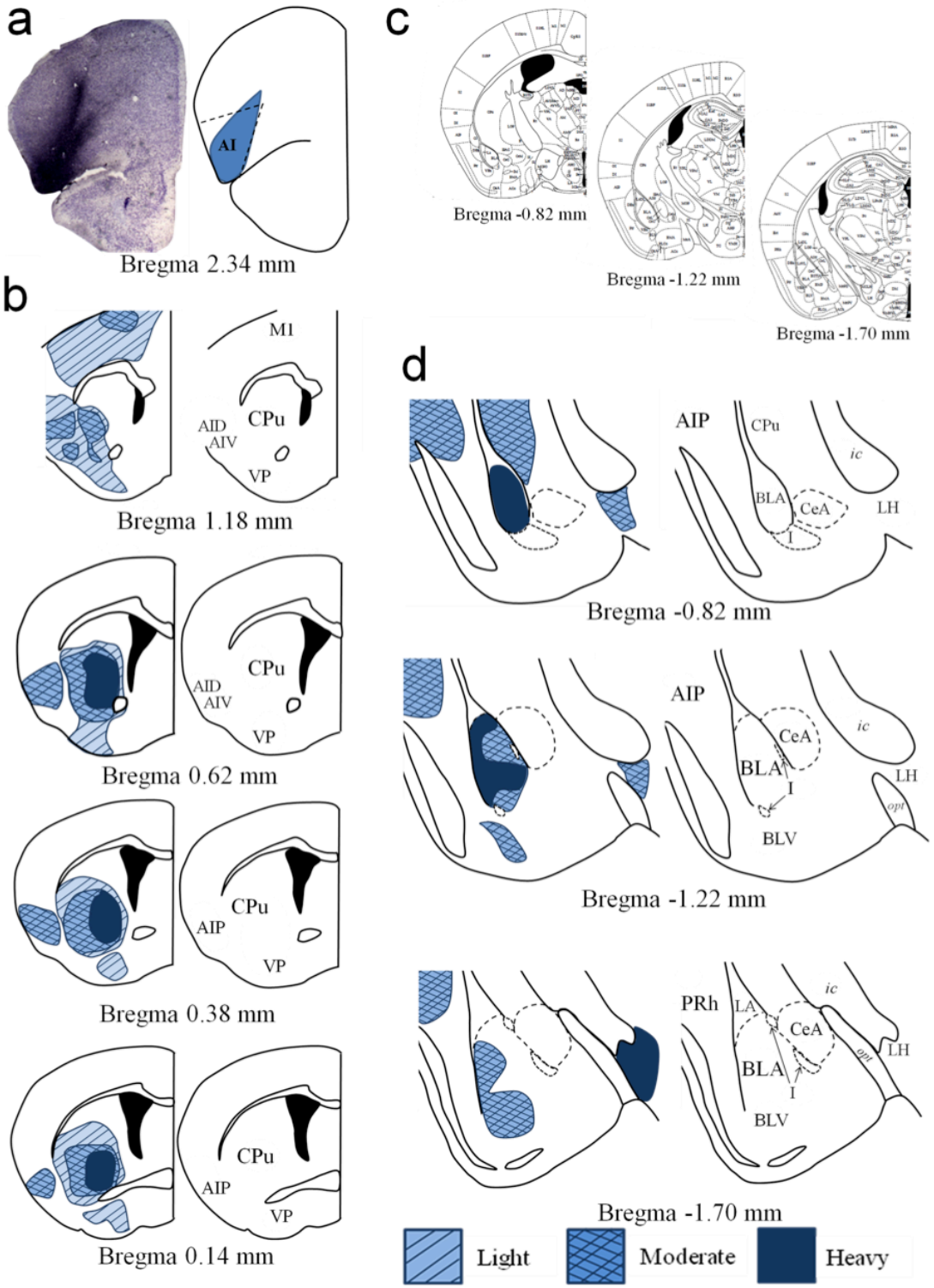
(a) A representative BDA infusion into the VLO and a drawing of the targeted area are shown (distance from bregma and estimated boundaries of regions based on Paxinos and Franklin, 2001). (b) The VLO innervates the dorsomedial and central striatum along the rostrocaudal axis. (c) Coronal amygdala sections from Paxinos and Franklin (2001) correspond to magnified depictions shown in (d). (d) Infusions of BDA into the VLO reveal innervation of the anterior BLA and the lateral wall of the posterior BLA, along with light innervation of the ITCs and moderate innervation of the dorsal endopiriform nucleus. Abbreviations: *AIP- posterior agranular insular cortex; AIV- ventral agranular insular cortex; BLA- basolateral amygdala; Cl- claustrum; CPu- caudate putamen; CeA- central amygdala; DEn- dorsal endopiriform nucleus; I- intercalated masses; ic- internal capsule; opt- optic tract.*

Figure 3-2: *Representative photomicrographs of the striatum and amygdala show innervation of the striatum and retrograde labeling of cell bodies in the BLA.*



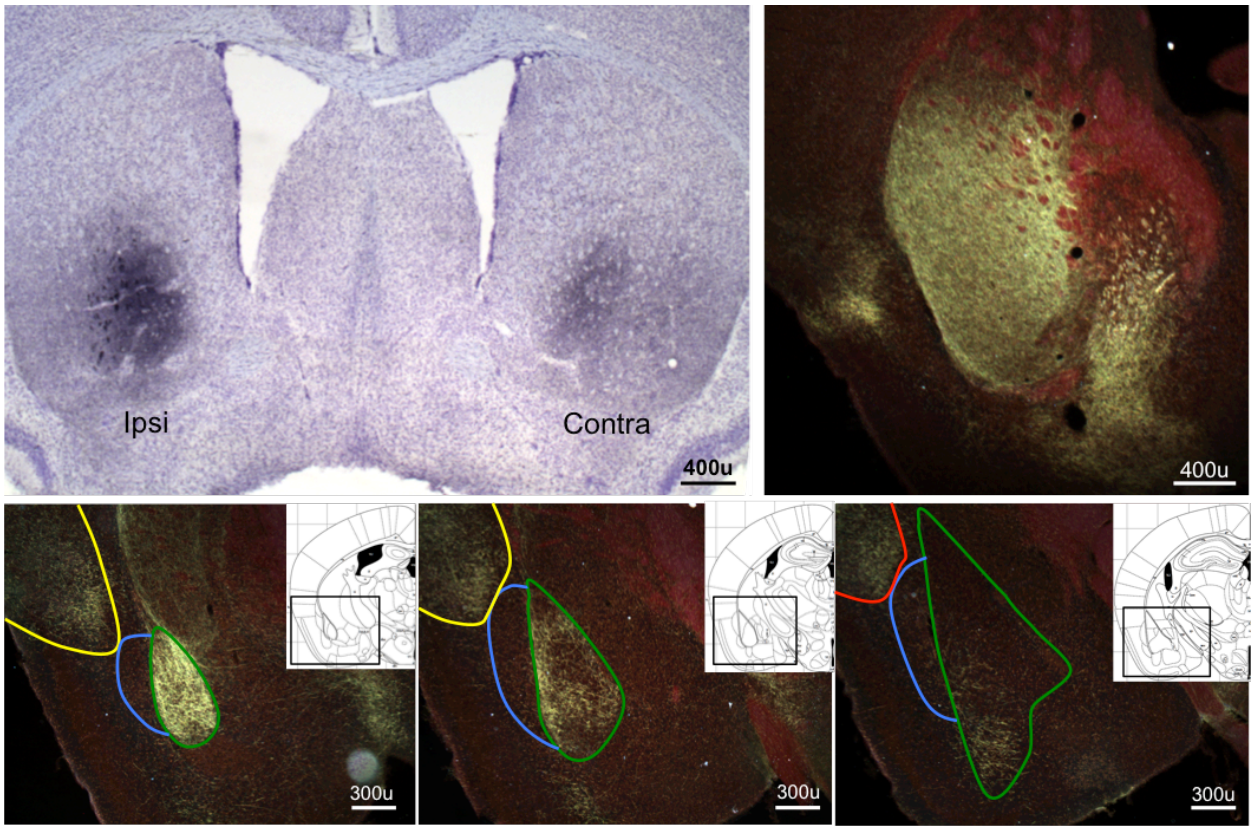
(a) Projections from the VLO enter the striatum through the external capsule and the gcc, and branch into fiber bundles. Corresponding image on left from Paxinos and Franklin (2001). (b) Magnification of labeled fiber bundles in the central zone of the striatum are shown – striatal innervation arises in part from axons branching off of these bundles. (c) Retrogradely filled cells in the BLA are evidence of reciprocal innervation between the VLO and the BLA.

Figure 3-3: *The DLO/AI innervates the lateral and ventral striatum, and sends topographically organized projections to the posterior AI, PRh, and BLA.*



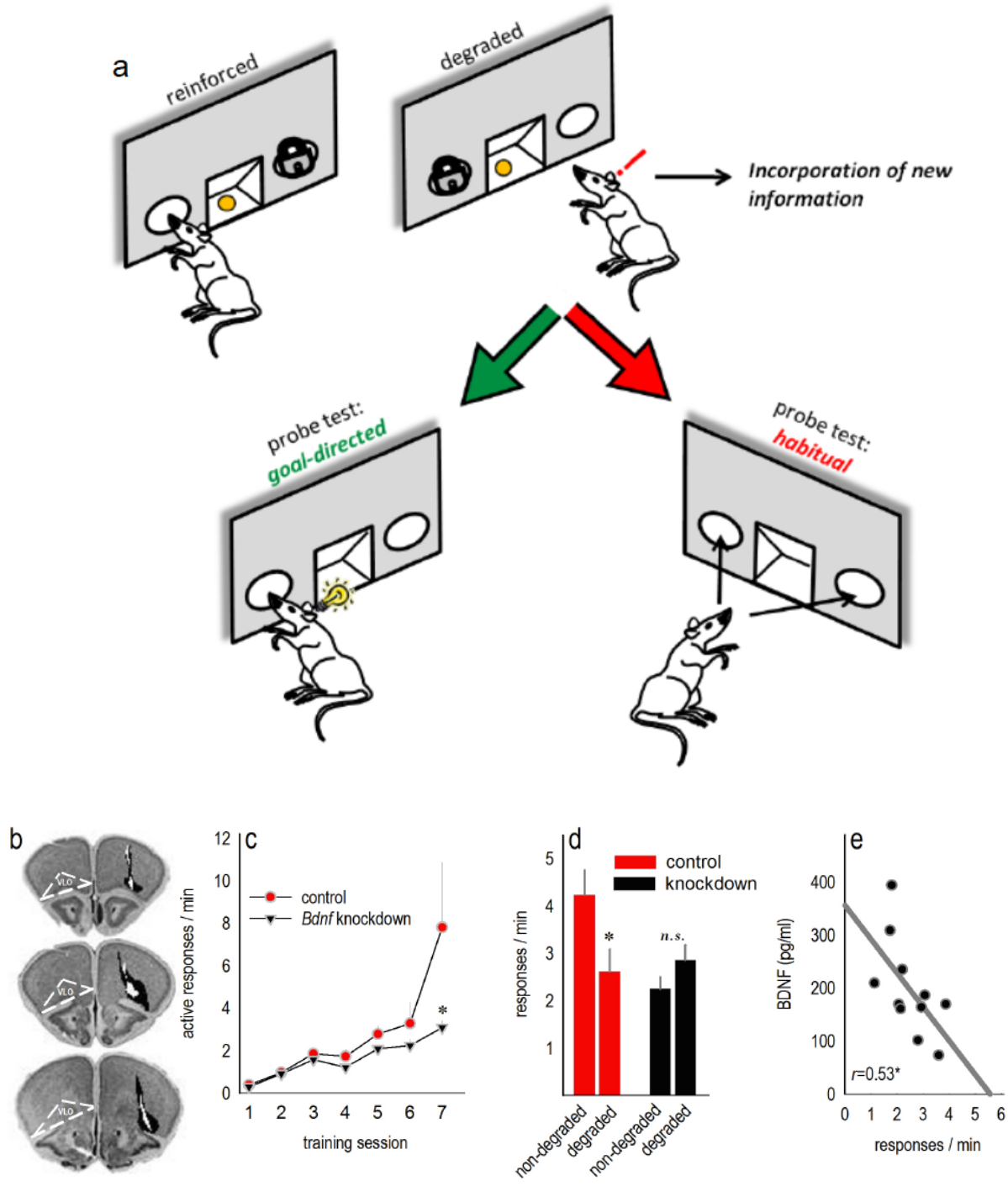
(a) A representative infusion of BDA into the DLO/AI and a drawing of the targeted area are shown. (b) BDA infusions into the DLO/AI illuminate heavy innervation of the ventral and lateral striatum, and reveal innervation of the posterior AI that is maintained along the rostrocaudal axis. (c) Coronal amygdala sections from Paxinos and Franklin (2001) correspond to the magnified depictions shown in (d). (d) The DLO/AI sends heavy projections to the anterior BLA. Projections are lighter in the posterior BLA, and preferentially terminate along the lateral wall. Innervation is also noted in the ventral BLA, as well as the posterior AI and the PRh. Abbreviations not defined in Fig.1: *AID- dorsal agranular insular cortex; BLV- basolateral amygdala, ventral part; LH- lateral hypothalamus; M1- primary motor cortex; PRh- perirhinal cortex; VP- ventral pallidum.*

Figure 3-4: *BDA infusions into the AI/DLO reveal bilateral rostrocaudal innervation of the striatum and topographically organized innervation of the PRh and BLA.*



(a) Projections from the DLO/AI to the striatum are bihemispheric, but labeling is heaviest in the hemisphere ipsilateral to the infusion site. (b) Infusions into the DLO/AI reveal rostrocaudal striatal innervation that culminates in heavy labeling in the posterior caudate. (c) The DLO/AI innervates the BLA, posterior AI, and PRh, but avoids the dorsal endopiriform nucleus. Green outline: BLA; blue outline: DEn; yellow outline: posterior AI; red outline: PRh. Inset: Corresponding images from Paxinos and Franklin (2001), with regions outlined in black.

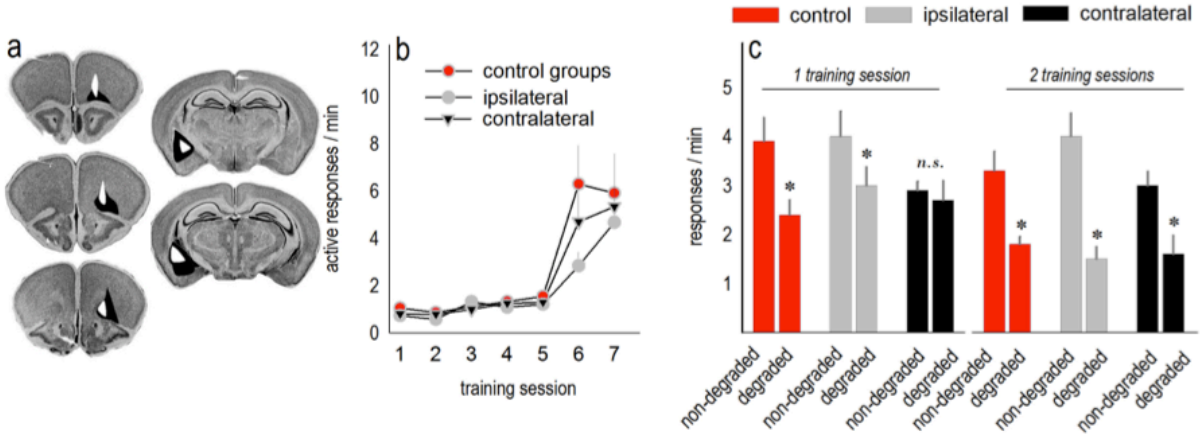
Figure 3-5: *VLO-selective Bdnf knockdown interferes with goal-directed action selection, resulting in reflexive habits.*



(a) A task schematic is shown. Mice are trained to generate two distinct responses. Then, the likelihood that one response will be reinforced is decreased. Preferential engagement of the

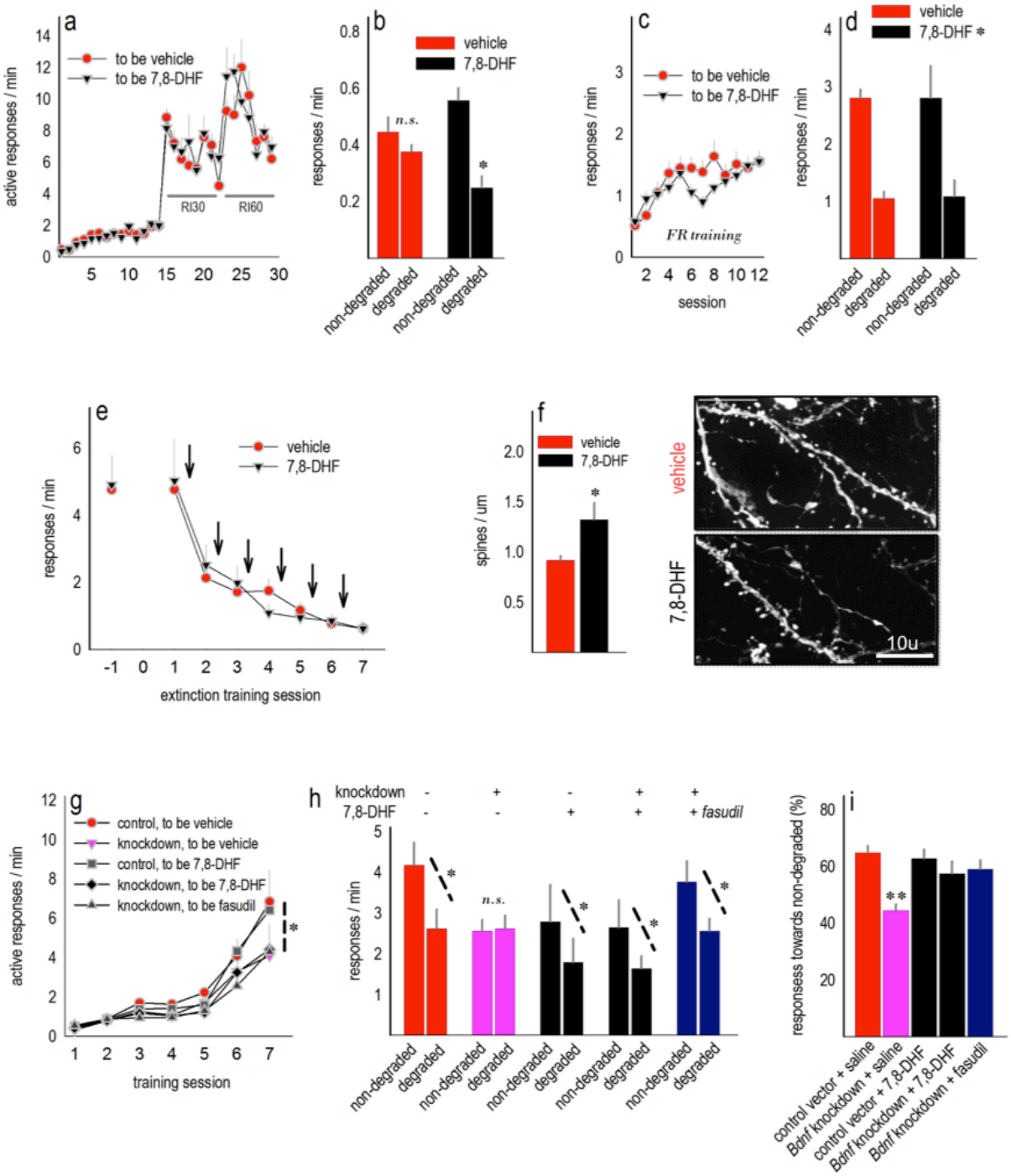
remaining response during a probe test is interpreted as goal-directed action selection, while engaging both responses equivalently — despite instrumental contingency degradation — is codified as habitual behavior. (b) *Bdnf* was knocked down in the VLO. Infusion sites are summarized on images from the Mouse Brain Atlas (Rosen et al., 2000). Black represents the largest viral vector spread, and white the smallest. Infusions were bilateral. (c) Mice were trained to nose poke for food reinforcers; *Bdnf* knockdown reduced response rates, particularly when the response requirement escalated from a continuous reinforcement to random interval schedule. Response rates represent total responses on both apertures. (d) Mice with *Bdnf* knockdown were also unable to select between actions that were more, *vs.* less, likely to be reinforced (non-degraded *vs.* degraded) following instrumental contingency degradation; instead, they engaged familiar response patterns, responding habitually on both. (e) BDNF expression in the downstream amygdala correlated with the degree of impairment following VLO-targeted *Bdnf* knockdown, with low BDNF associated with robust responding on the 'degraded' nose poke aperture. Symbols and bars represent means+SEMs, except in (e) where each symbol represents a single mouse. *$p<0.05$.

Figure 3-6: *Functional disconnection of the VLO and amygdala results in reflexive habits.*



(a) Histological representations of unilateral cortical viral vector infusions and amygdalar lesions are transposed onto images from the Mouse Brain Atlas (Rosen et al., 2000). Black represents the largest and white the smallest. (b) Mice were trained to nose poke for food reinforcers; there were no differences between groups. (c) Mice with asymmetric infusions were, however, insensitive to instrumental contingency degradation. With an additional training session, mice ultimately were able to develop outcome-directed response strategies, indicating that contralateral infusions delayed, but did not block, action-outcome conditioning. Symbols and bars represent means+SEMs, *p<0.05.

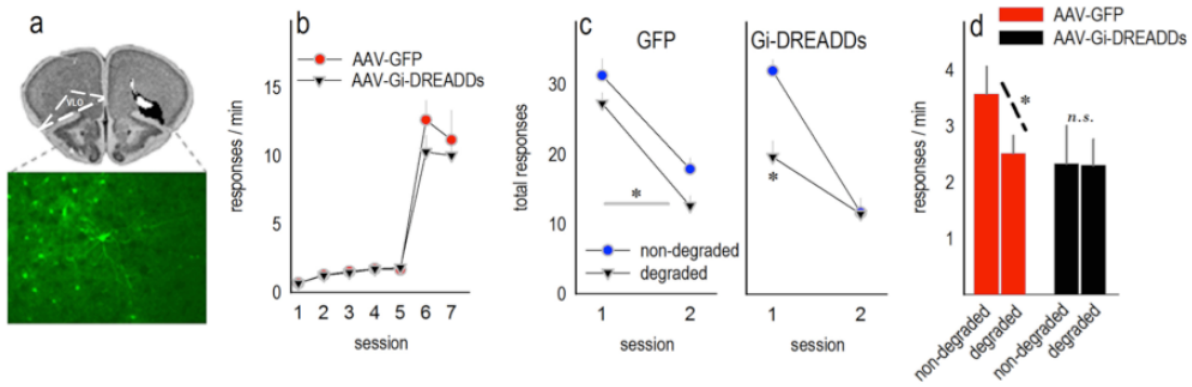
Figure 3-7: *7,8-DHF rescues goal-directed decision-making and regulates VLO dendritic spines.*



(a) Intact mice were extensively trained to respond for food reinforcement. Escalating random interval schedules are indicated. (b) The TrkB agonist, 7,8-DHF, preserved sensitivity to action-

outcome contingency degradation, despite extended response training, while control mice developed habits as expected. (c) A separate group of mice was trained to nose poke using a continuous reinforcement schedule. (d) As a control, we then confirmed that 7,8-DHF had no effects when mice would be expected to engage in goal-directed decision-making strategies. (e) Additionally, 7,8-DHF had no effects on extinction conditioning when the reinforcer was withheld entirely. Each arrow represents an injection following the test session. (f) Layer V dendritic spines were imaged and enumerated in these mice. 7,8-DHF increased spine density in the VLO. Representative dendritic branches are adjacent. (g) Next, separate mice with VLO-targeted *Bdnf* knockdown were trained to respond for food reinforcers. Treatment groups were designated by matching mice based on response acquisition curves. (h) *Bdnf* knockdown induced inflexible habit-like responding as expected, but 7,8-DHF rescued sensitivity to instrumental contingency degradation, as did the Rho-kinase inhibitor fasudil. (i) The same data are represented as the percentage of total responses directed towards the aperture most likely to be reinforced. The pink bar at ~50% indicates that *Bdnf* knockdown mice respond at chance levels, while 7,8-DHF or fasudil treatment restored selective responding. Bars and symbols represent means+SEMs. *$p<0.05$ as indicated, **$p<0.04$ relative to all other groups. “FR” refers to fixed ratio 1 (continuous reinforcement) training.

Figure 3-8: *Gi-DREADD-mediated silencing of the VLO results in stimulus-response habits.*



(a) Mice were infused bilaterally with AAV-GFP or AAV-hM$_4$D(Gi)-mCitrine. Infusions sites are summarized, and mCitrine-expressing neurons are shown, providing evidence of DREADD infection of the VLO. (b) In the absence of CNO, there were no differences between groups in response acquisition. (c) When the inert ligand was paired with degradation of the instrumental contingency, control GFP-expressing mice subsequently preferentially generated the response more likely to be reinforced over 2 x 5-min bins. By contrast, Gi-DREADD-expressing mice initially preferred the 'non-degraded' response, but then responding on each nose poke equalized. (d) The same findings are represented in bar graph form, again showing that Gi-DREADD-expressing mice responded non-selectively. Symbols and bars represent means+SEMs, *p<0.05.

Chapter 4:

The orbitofrontal cortex regulates behavioral flexibility in both appetitive and aversive domains

4.1 Context and Author's Contribution

The following chapter presents evidence that LTP in excitatory neurons in the VLO is necessary for behavioral flexibility in both appetitive action-outcome and aversive stimulus-outcome conditioning tasks. These findings suggest that the VLO is uniquely sensitive to the likelihood of outcome delivery, independent of the emotional valence of the outcome, or whether the task is based in instrumental or classical conditioning. The dissertation author contributed to the chapter by designing and conducting the majority of the experiments, analyzing data, and writing the manuscript under the guidance of Drs. Shannon Gourley, Kerry Ressler, and Donald Rainnie. Dr. Chenchen Li conducted the electrophysiology experiments and assisted in analyzing the associated data.

4.2 Abstract

The orbitofrontal cortex (OFC) encodes changes in the predictive relationship between reward-related stimuli and associated outcomes, but less is known about its role in detecting changes in *response*-outcome associative contingencies. Furthermore, the majority of OFC literature in rodents has focused on appetitive conditioning tasks — little is known about its role in processing aversive outcomes, despite evidence implicating the OFC in fear-related psychopathologies. Here, we delivered CaMKII-driven Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in the ventrolateral subregion of the OFC (VLO). Mice were then trained to generate two food-reinforced responses. Next, the likelihood that one response would be reinforced was reduced, and the DREADD-activating ligand Clozapine-N-oxide (CNO) was paired with this training. A probe test conducted 24 hours later was used to determine whether mice expressed goal-directed, or habitual, response strategies.

Subsequently, mice were subjected to Pavlovian fear conditioning, and CNO was administered in conjunction with extinction training. Extinction retention tests were conducted in the absence of further CNO treatment. The physiological effects of DREADD activation were determined in slice preparations via standard patch clamp recording techniques. Gi-DREADD activation in the VLO in concert with response-outcome contingency degradation obstructed goal-directed decision-making. Moreover, activation during fear extinction training prevented extinction retention. Single-cell recordings indicated that Gi-DREADD activation attenuated long-term potentiation (LTP) induction but did not impact intrinsic membrane properties. Together, these data suggest that LTP in the VLO is necessary for stable encoding of outcome-based learning and memory in both appetitive and aversive domains.

4.3 Introduction

Successfully navigating a changing environment requires organisms to form associations between stimuli or behaviors and specific outcomes, and to modify these associations when appropriate. These processes are thought to involve the orbitofrontal prefrontal cortex (OFC), as well as limbic structures like the amygdala, which shares rich reciprocal connections with the OFC (McDonald, 1991; McDonald et al., 1996). For example, when an expected outcome is not delivered upon presentation of a formerly predictive stimulus or the completion of a learned response, the OFC is thought to detect this error and facilitate new learning, for instance by "teaching" the downstream basolateral amygdala (BLA) to modify the original association (Delamater, 2007; Morrison et al., 2011). This development of new associations, and the ability to engage new behavioral response strategies accordingly, is an important aspect of goal-directed decision-making.

The inability to modify or override previously learned associations when they are no longer valid or otherwise behaviorally advantageous is associated with a broad range of psychopathologies—for instance, in unremitting drug seeking in addiction, in repetitive acts of compulsion, and in extinction-resistant fear expression in Post-traumatic Stress Disorder (PTSD). Accordingly, abnormal OFC structure and function are associated with obsessive-compulsive disorder (Rauch et al., 1994) and phobias that fail to extinguish (Tillfors et al., 2001), as well as addiction and PTSD (Volkow et al., 2011; Jackowski et al., 2012). Notably, OFC dysfunction is in some cases responsive to cognitive behavioral therapy (Kennedy et al., 2007) and antidepressants (Kennedy et al., 2007; Fani et al., 2011), making it a promising target in understanding and improving treatment approaches for a broad spectrum of disorders.

When behaviors are carried out reflexively, independent of the likelihood that they will be reinforced with an expected outcome, these behaviors are considered "habitual". Lesion studies recently indicated that the ventrolateral OFC (VLO) is essential to suppressing habit-based modes of response, thus facilitating flexible decision-making (Gourley et al., 2013c; Gremel and Costa, 2013). However, these findings remain somewhat unexplored, and relatively little is known about how the VLO regulates fear expression and extinction conditioning in mice or rats. This is despite suggestions that pathological failures in fear extinction can be conceptualized as habit-like, as well as considerable homology between the rodent and primate OFC (Preuss, 1995) and the broad utility of rodent models in understanding causal relationships between biology and behavior. A recent study demonstrated that pharmacological inactivation of the medial OFC (MO) in rats decreases conditioned fear (Rodriguez-Romaguera et al., 2015); however, to our knowledge, the role of the VLO remains unclear.

Here we use Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to selectively impair VLO function in an inducible fashion during ***1)*** instrumental contingency degradation, and ***2)*** the extinction of conditioned fear. Finally, we used single-cell patch clamp recording to establish the physiological effects of Gi-DREADD activation on neuronal activity.

4.4 Materials and Methods

4.4.1 Subjects.

≥6 week-old male C57BL/6 mice (Jackson Labs) were used in all experiments. Mice were maintained on a 12-hour light cycle (0700 on) and allowed *ad libitum* access to food and water, except during instrumental conditioning when body weights were maintained at ~90% of baseline in order to motivate responding. All procedures were approved by the Emory University IACUC.

4.4.2 Surgery.

Mice were anesthetized with an intraperitoneal (i.p.) injection of Ketaset/Dexdormitor (75 mg/kg and 1 mg/kg, respectively). For viral vector experiments, AAV5-CaMKII-HA-hM$_4$D(Gi)-IRES-mCitrine or AAV5-CaMKII-GFP (UNC Viral Vector Core) were infused bilaterally into the VLO at a volume of 0.5 μl/side. Coordinates were: AP+2.6, ML±1.2, DV-2.8. Infusions were delivered at a rate of 0.05 μl/minute and the needle was left in place for an additional 5 minutes. Following infusions, the scalp was sutured and mice were revived with an i.p. injection of Antisedan (1 mg/kg). Mice were allowed a minimum of 2 weeks for recovery and full viral vector expression before behavioral training or physiological experiments.

4.4.3 Drug.

Clozapine N-Oxide (CNO) was purchased from Sigma-Aldrich. It was dissolved in a 2% dimethyl sulfoxide (DMSO) solution and administered i.p. at a dose of 1 mg/kg.

4.4.4 Instrumental conditioning.

Mice were trained to nose poke for food reinforcement (20 mg grain-based pellets; Bioserv) using illuminated Med-Associates conditioning chambers equipped with two nose poke recesses and a separate food magazine. Training was initiated using a fixed ratio (FR) 1 schedule of reinforcement; mice could earn up to 30 pellets for responding on each of 2 active apertures, and the sessions ended when all potential 60 pellets had been delivered, or at 135 minutes. Following 5 days of FR1 training, mice were shifted to a random interval 30 second schedule of reinforcement for 2 days; again, the session was terminated when all 60 pellets had been earned, or at 135 minutes.

Following training, mice were tested for sensitivity to response-outcome contingency degradation during 2 25 minute sessions. As previously described (Gourley et al., 2012b; Gourley et al., 2013b; Swanson et al., 2015), during the 'reinforced' session, one of the nose poke apertures was occluded, and responding on the remaining aperture was reinforced according to a variable ratio 2 schedule of reinforcement. During the 'degraded' session, the opposite aperture was available, but inactive. Instead, reinforcers were delivered independently of animals' responding, at a rate matched to each subject's reinforcement rate the previous day. This manipulation "degrades" the predictive association between this response and the food reinforcer, and thus, one response becomes significantly more predictive of reinforcement than

the other. Mice were administered an i.p. injection of CNO either 30 minutes prior to the 'degraded' session or immediately after, to target the acquisition of conditioning, and the putative consolidation period (Gourley et al., 2013b), respectively.

To determine whether mice could use information regarding response-outcome contingencies to make flexible, goal-directed decisions, mice were returned to the chambers 24 hours later and allowed access to both apertures in a 10 minute probe test conducted in extinction. In one experiment, a second probe test was conducted the following day. Preferential engagement of the response most likely to result in reinforcement is considered goal-directed, whereas equivalent engagement of both familiar responses is considered habitual (Yin et al., 2008; Balleine and O'Doherty, 2010).

4.4.5 Fear conditioning and extinction.

Following instrumental contingency degradation testing, mice were returned to *ad libitum* feeding for at least a week prior to fear experiments. For two days prior to fear conditioning, mice were habituated to the conditioning chambers (Med-Associates). Habituation sessions were conducted by placing mice in the chambers for 15 minutes each day in the absence of any stimuli. Auditory fear conditioning was then conducted; this 8 minute session consisted of 5 presentations of a 30 second, 6kHz tone (conditioned stimulus — CS) co-terminating with a 1 second, 0.6 mA footshock (unconditioned stimulus — US). Extinction training was then conducted in a novel context, and consisted of 15 presentations of the CS in the absence of the US. This session was 17 minutes long and began with a 3 minute habituation period before the first CS presentation. An i.p. injection of CNO was administered 30 minutes prior to extinction training. Extinction retention tests were conducted in the absence of CNO, and again consisted of

15 non-reinforced presentations of the CS. The percent of time mice spent freezing — a measure of conditioned fear — in the presence of the CS was determined using FreezeFrame and FreezeView software (Coulbourn Instruments, #ACT-100).

4.4.6 Histology.

Mice were deeply anesthetized and transcardially perfused with chilled 4% paraformaldehyde. Brains were rapidly removed and stored in chilled 4% paraformaldehyde for 24 hours, followed by immersion in 30% sucrose for a minimum of 48 hours. The brains were then sectioned at 55 μm on a freezing microtome held at -15°C and stored in cryoprotectant until further processing. Every third section from the prefrontal cortex was mounted on Fisherbrand Superfrost Plus Microscope Slides. Slides were coverslipped using Vectashield Mounting Media and imaged for GFP (controls) or mCitrine (Gi-DREADDs) on a Nikon Eclipse inverted microscope.

4.4.7 Electrophysiology.

Slice preparation

350 μm sections containing the VLO were obtained as previously described (Rainnie, 1999). Briefly, mice were anesthetized with isoflurane, and brains were rapidly dissected and immersed in a 4°C 95-5% oxygen/carbon dioxide oxygenated "cutting solution", made up of NaCl xmM, NaHCO3, KCl, KH2PO4, MgCl2, CaCl2, glucose, and supplemented with kynurenic acid. VLO-containing slices were cut using a Leica VTS-1000 Vibratome (Leica Microsystems Inc.), and maintained at 37°C in oxygenated cutting solution for at least 50 minutes prior to being transferred to artificial cerebrospinal fluid (ACSF), made up of NaCl

xmM, NaHCO3, KCl, KH2PO4, MgCl2, CaCl2, glucose. Sections were maintained in ACSF at least 30 minutes prior to recording.

Patch clamp recording

Patch clamp recordings and LTP induction were conducted as previously described (Li et al., 2011a). Briefly, following tissue preparation 350 μm brain sections containing the VLO were transferred to a submersion-type recording chamber mounted on a fixed stage Leica DMLFS microscope (Leica Microsystems Inc.) and continuously perfused by gravity-fed oxygenated ACSF at 32°C with a flow rate of 1-2 ml/minute. Neurons were identified using differential interference contrast (DIC) optics and infrared (IR) illumination with an IR sensitive CCD camera (Orca ER). Thin-walled borosilicate glass patch electrodes, which had a resistance of 4-6 MΩ, were filled with (in mM): K-gluconate (130), KCl (2), HEPES (10), MgCl2 (3), K-ATP (2), NaGTP (0.2), and phosphocreatine (5), adjusted to pH 7.3 with KOH, and having an osmolarity of 280-290 mOsm. AAV transfected VLO neurons were first identified as being either GFP- or mCitrine-positive using fluorescence microscopy. Neurons were then visualized for recording using DIC microscopy in combination with a 40x water immersion objective and displayed in real time on a computer monitor. Data acquisition and analysis were performed using a MultiClamp700B amplifier in conjunction with pClamp10.0 software and a DigiData 1320A AC/DA interface (Molecular Devices). Whole cell patch clamp recordings were obtained, and recorded voltages were low-pass filtered at 5 kHz and digitized at 10-20 kHz.

Excitatory postsynaptic currents (EPSCs) onto VLO neurons were evoked by placing a concentric bipolar stimulation electrode (FHC) approximately 500 μm from the recorded neuron, close to the fiber tract of the forceps minor of the corpus callosum immediately adjacent to the VLO. To isolate evoked EPSCs, 50 μM picrotoxin was added to the patch solution to block

$GABA_A$ currents exclusively in the recorded neuron. Sections were continuously perfused with oxygenated ACSF (32°C) containing the selective $GABA_B$ receptor antagonist, CGP36742 (5 μM). This recording configuration allowed stable recording of isolated EPSCs without contamination from epileptiform, recurrent EPSCs.

EPSCs (adjusted to 30% of maximal response) were evoked at 0.05 Hz, a 10 minute baseline period was recorded, and recordings continued for at least 40 minutes after LTP induction. Initial EPSC amplitudes were normalized to the average of the baseline EPSC amplitude. To induce LTP, a 5x high frequency stimulation (HFS) LTP protocol was employed, which consisted of 5 trains of 100 Hz stimulation delivered for 1 second, with a 20 second interval between each train. As previously reported, this form of LTP is NMDA-receptor-dependent (Woo et al., 2003). During EPSC measurement a negative DC holding current was injected into the neurons to maintain the membrane potential at -70 mV, except during HFS when the potential was adjusted to -60 mV to facilitate spike firing.

To calculate the paired-pulse ratio (PPR), two EPSCs were evoked with an inter-stimulus-interval of 50 ms, and PPR was calculated as ($eEPSC_1/eEPSC_2$), where $eEPSC_1$ and $eEPSC_2$ represent the amplitude of the first and the second eEPSC, respectively. PPR was measured before, immediately after, as well as 10 minutes and 30 minutes after 5xHFS.

Drug application

CNO (100 μM) was applied in the ACSF using continuous gravity fed bath application. Final concentration of DMSO was no more than 0.1%.

4.4.8 Statistical analysis.

Two-tailed parametric statistical analyses with $\alpha \leq 0.05$ were performed using SigmaStat v.3.1 or SPSS. Tukey's post-hoc t-tests were applied in the event of significant interaction effects and are indicated graphically. Correlations reflect linear regression analyses drawn from control GFP-expressing mice. Values lying >2 standard deviations outside of the mean were considered outliers and excluded.

4.5 Results

4.5.1 The VLO is necessary for goal-directed decision-making.

The selective innervation of the dorsal striatum and the BLA by the VLO (see again, Chapter 3) suggests that it is ideally situated to regulate associative conditioning in both appetitive and aversive domains. To test this, we infused CaMKII-driven viruses expressing either GFP (controls) or Gi-DREADDs bilaterally into the VLO, allowing for selective and controlled suppression of activity in local excitatory neurons by systemic administration of CNO. Viral vector infection was largely restricted to the VLO, avoiding spread to the MO and the DLO/AI (fig.4-1a); minimal and maximal spread was similar to that demonstrated in fig.3-8a.

We trained the mice to generate two instrumental responses, each of which resulted in delivery of a food reinforcer. Following response acquisition, the response-outcome contingency associated with *one* of the responses was degraded by providing reinforcers associated with this response non-contingently, while responding on the other aperture remained reinforced (fig.4-1b). CNO was administered to both groups either 30 minutes prior to "degradation training" (Experiment 1) or immediately following (Experiment 2). Mice were subsequently given a brief probe test conducted in extinction. Preferential engagement of the response most likely to result

in reinforcement is codified as goal-directed action selection, whereas equivalent engagement of both responses is considered habitual (Yin et al., 2008; Balleine and O'Doherty, 2010).

In Experiment 1, all mice acquired the instrumental responses, with no difference in response rates between groups [interaction $F_{(6,108)}$=1.4, p=0.2; main effect of virus $F_{(1,18)}$=1.3, p=0.3] (fig.4-1c). When CNO was administered prior to a session wherein one response-outcome contingency was "degraded," control mice subsequently preferentially generated the response most likely to be reinforced in a goal-directed fashion. Meanwhile, Gi-DREADD-expressing mice failed to develop a preference, generating both familiar responses equivalently in a habitual manner [interaction $F_{(1,18)}$=5.0, p=0.04] (fig.4-1d).

In Experiment 2, mice again acquired the instrumental responses, with no group differences [interaction and main effect F<1] (fig.4-1e). In this case, CNO was administered immediately following instrumental contingency degradation in order to attempt to disrupt the consolidation of new conditioning. During a subsequent probe test, all mice initially preferentially generated the response that was most likely to be reinforced in a goal-directed fashion [main effect of response $F_{(1,12)}$=21.4, p=0.001; interaction F<1]. However, when an identical probe test was conducted the following day, control mice again preferentially generated the response that was most likely to be reinforced, while the expression of goal-directed action selection strategies decayed in the Gi-DREADD group, such that these mice responded in a non-selective, habitual fashion [interaction $F_{(1,12)}$=6.4, p=0.03] (fig.4-1f). This suggests that neuroplasticity in the principal excitatory neurons of the VLO is necessary for the stable consolidation of new response-outcome associations.

4.5.2 The VLO is necessary for the retention of fear extinction.

We next trained mice in an auditory fear conditioning task. Following conditioning, we conducted an extinction training session, with the hypothesis that the VLO may be involved in encoding new information, specifically, that a foot shock-associated CS is no longer predictive of the US, the shock itself. CNO was administered 30 minutes prior to this session. Extinction retention tests were conducted drug-free to determine how well the mice extinguished the CS-US contingency (fig.4-2a).

All mice quickly acquired conditioned fear, evidenced by increased freezing behavior over the 5 CS-US presentations. No differences in freezing scores were noted between groups during this acquisition phase [interaction $F_{(4,40)}$=1.8, p=0.15; main effect of virus F=1] (fig.4-2b). When CNO was administered prior to extinction training, no within-session differences were identified between the control and Gi-DREADD groups; in other words, CNO did not impact the extinction of conditioned fear during an initial test [interaction F<1; main effect of virus $F_{(1,10)}$=1.2, p=0.3] (fig.4-2c). However, when mice were again tested drug-free the following days, the Gi-DREADD group demonstrated significant deficits in extinction retention, continually expressing higher levels of freezing in response to the CS presentations than control mice [Retention1, main effect of virus $F_{(1,10)}$=8.8, p=0.01 (fig.4-2d)] [Retention2, main effect of virus $F_{(1,10)}$=19.2, p=0.001 (fig4-2e)] [Retention3, main effect of virus $F_{(1,10)}$=6.6, p=0.03 (fig.4-2f)] [overall virus x day interaction $F_{(4,40)}$=3.6, p=0.01 (fig.4-2g)].

4.5.3 Instrumental response strategies correlate with fear extinction retention.

We next compared individual performances in both instrumental contingency degradation and extinction retention tests in mice from Experiments 1 and 2, using the percentage of total responses directed towards the non-degraded contingency during the probe test and average

freezing across all tones in the first extinction retention test as metrics of "flexible" behavior. Increased preferences for the response that was predictive of reinforcement were associated with decreased freezing during extinction retention tests [r^2=0.4, p=0.01] (fig.4-3). In other words, the expression of goal-directed decision-making strategies was associated with the successful extinction of conditioned fear.

4.5.4 Gi-DREADD activation increases the threshold for LTP.

To determine the physiological effects of CNO application on Gi-DREADD infected VLO neurons, we generated sections from a separate cohort of mice expressing GFP or Gi-DREADD-mCitrine in the VLO. We then electrically stimulated the forceps minor of the corpus callosum and recorded pharmacologically isolated excitatory postsynaptic currents (EPSCs) from nearby infected VLO neurons (identified by the expression of GFP or mCitrine) (fig.4-4a). Baseline electrophysiological recordings of the intrinsic membrane properties of VLO neurons collected before and after bath application of CNO (100 μM) showed no differences in the resting membrane input resistance or action potential firing rate (fig.4-4b), suggesting that the behavioral effects we report were not due to Gi-induced membrane potential hyperpolarization or other disruptions of intrinsic membrane properties caused by activation of the Gi-DREADD.

We next tested the effects of Gi-DREADD activation on LTP induction in VLO neurons. To test this, evoked-EPSCs were recorded for 15 minutes prior to CNO application to establish a baseline measure of EPSC amplitude. Following a 10 minute bath application of CNO (100 μM), GFP or Gi-DREADD infected neurons (one neuron used from each animal) were subjected to five bursts of high frequency stimulation (HFS; 1 sec at 100 Hz) to induce LTP. Subsequent to HFS, EPSCs were recorded for at least 40 minutes to determine the effect of CNO application on

LTP induction. Compared to GFP expressing VLO neurons, Gi-DREADD expressing neurons exposed to CNO showed significantly attenuated LTP magnitude in response to HFS [interaction $F_{(10,170)}=3.7$, $p<0.001$] (fig.4-4c). Fig.4-4d shows normalized EPSC amplitude for each data point at 30 minutes following HFS.

4.6 Discussion

Appetitive and aversive conditioning are frequently studied in isolation from one another. However, many of the processes and neural correlates necessary for associative conditioning are common to both positive-valenced (reward-based) learning and negative-valenced (fear-based) learning (for instance, see (Jones et al., 2008; Choi et al., 2012; Heldt et al., 2014)). Here we demonstrate that plasticity in the one region of the OFC, the VLO, is necessary for consolidating changes to familiar associations, irrespective of valence. Specifically, DREADD-mediated inhibition of plasticity in the VLO impaired the stable consolidation of new information regarding the predictive relationship between an action and an outcome, disrupting flexible, goal-directed decision-making. Likewise, inhibition during extinction conditioning following Pavlovian fear conditioning prevented the retention of extinction conditioning, resulting in an inflexible maintenance of CS-elicited freezing.

We utilized here two forms of outcome-based conditioning: response-outcome contingency degradation, in which the predictive relationship between a *behavior* and the associated *reinforcer* is violated, and Pavlovian fear conditioning, in which a previously-neutral *stimulus* is associated with a particular *outcome*. Despite clear procedural and conceptual differences between response-outcome and stimulus-outcome conditioning, both types of conditioning require: ***1)*** the initial formation of an outcome-based association, and ***2)*** subsequent

modification of that association if the likelihood that the outcome will be delivered changes. We report that the VLO regulates outcome-based learning and memory by consolidating new information about previously acquired associations. Furthermore, our findings support a valence-free model in which the VLO encodes the *likelihood* of outcome delivery, independent of whether learning is driven by appetitive or aversive motivations.

A brief note on VLO projection patterns.

We and others have previously reported that VLO projections target basal ganglia and limbic regions involved in decision-making and associative learning and memory (McDonald et al., 1996; Groenewegen et al., 1997; Schilman et al., 2008) (see again, **Chapter 3**). Specifically, the VLO preferentially targeted the central region of the dorsal striatum, with heaviest innervation considerably ventral to the corpus callosum but nonetheless sparing the ventral striatum (see fig.3-1), consistent with previous reports (Berendse et al., 1992; Groenewegen et al., 1997; Schilman et al., 2008). This area of the dorsal striatum targeted by the VLO is involved in goal-directed action selection (Yin et al., 2008; Gourley et al., 2013b), and additionally, instrumental conditioning — that is, associating a behavioral response with the delivery of a reinforcer — stimulates immediate-early gene expression in this region (Yin et al., 2008; Maroteaux et al., 2014). Also, we have previously reported that disconnection of the VLO and this central region of the dorsal striatum disrupts goal-directed decision-making in the same instrumental contingency degradation task used here (Gourley et al., 2013c). Within the amygdala, VLO projections innervate the BLA and largely spare the CeA (see fig.3-1). This is again consistent with previous findings in rodents (McDonald et al., 1996; Groenewegen et al., 1997). The BLA regulates both goal-directed decision-making (Balleine et al., 2003) and the

acquisition and extinction of conditioned fear (Davis, 1993; Maren, 1999). Selective targeting of the dorsal striatum and the BLA by the VLO suggests that the VLO is ideally situated to regulate associative conditioning and behavioral flexibility.

The VLO regulates goal-directed action selection.

We find that impairing neuroplasticity in the VLO interferes with new learning regarding the predictive relationship between actions and their outcomes. This can be attributed at least in part to impaired consolidation of response-outcome learning and memory, given that both pre- *and post*- training CNO injections were sufficient to destabilize goal-directed action selection in subsequent probe tests, leading to a reliance on inflexible, habitual response strategies. We have previously reported that knocking down the neuroplasticity-associated gene *Brain-derived neurotrophic factor* (*Bdnf*) selectively in the VLO also disrupts goal-directed decision-making (Gourley et al., 2013c). The same study showed that knockdown of *Bdnf* in the VLO resulted in reduced expression of BDNF protein in both the dorsal striatum and amygdala. We proposed that anterograde transport of BDNF from VLO-originating projection neurons to these downstream sites facilitate the expression of flexible, goal-directed action selection. In other words, outcome-based decision-making may be regulated not only by local plasticity in the VLO, but also by VLO-mediated plasticity in the striatum and amygdala.

Notably, systemic administration of cocaine immediately following instrumental contingency degradation training disrupts subsequent goal-directed decision-making, as with CNO administration in Gi-DREADD-expressing mice here (Gourley et al., 2013b). Cocaine also decreases the expression and activity of synaptic and cytoskeletal markers in the dorsal striatum (Gourley et al., 2013b) and eliminates dendritic spines in the OFC, including the VLO subregion

(DePoy and Gourley, 2015). Moreover, cocaine simplifies dendrite arbors in the OFC, and these structural changes correlate with the development inflexible, habit-like behaviors in an instrumental reversal task (DePoy et al., 2014). Together, these findings further support a model in which plasticity within the OFC and its target regions is necessary for successful consolidation of new information about relationships between actions and their outcomes.

The VLO regulates the extinction of conditioned fear.

As discussed above, the rodent VLO appears to be required for appetitive conditioning and flexible reward-related decision-making (Dalley et al., 2004; Gourley et al., 2012a; Gourley et al., 2013c; Swanson et al., 2015). To our knowledge, however, its influence on fear conditioning and extinction has not yet been established. We report that DREADD-mediated disruption of VLO plasticity causes significant deficits in the retention of extinction conditioning. Notably, CNO was administered prior to extinction training, and yet, it had no effects during this initial session. Rather, mice failed to inhibit freezing when subsequently exposed to the CS, indicating that plasticity in VLO excitatory neurons is necessary for successful consolidation, but not necessarily the initial acquisition, of extinction conditioning. We suggest that DREADD-mediated interference with neuroplasticity in the VLO prevents the stabilization of the extinction memory, which would normally occur during the consolidation period. Meanwhile, other neural substrates, for instance the infralimbic cortex (IL), may more immediately facilitate the suppression of conditioning freezing during extinction training itself. In this way, the IL and the VLO may function synergistically to promote the acquisition and consolidation of extinction.

Gi-DREADD activation inhibits LTP in the VLO.

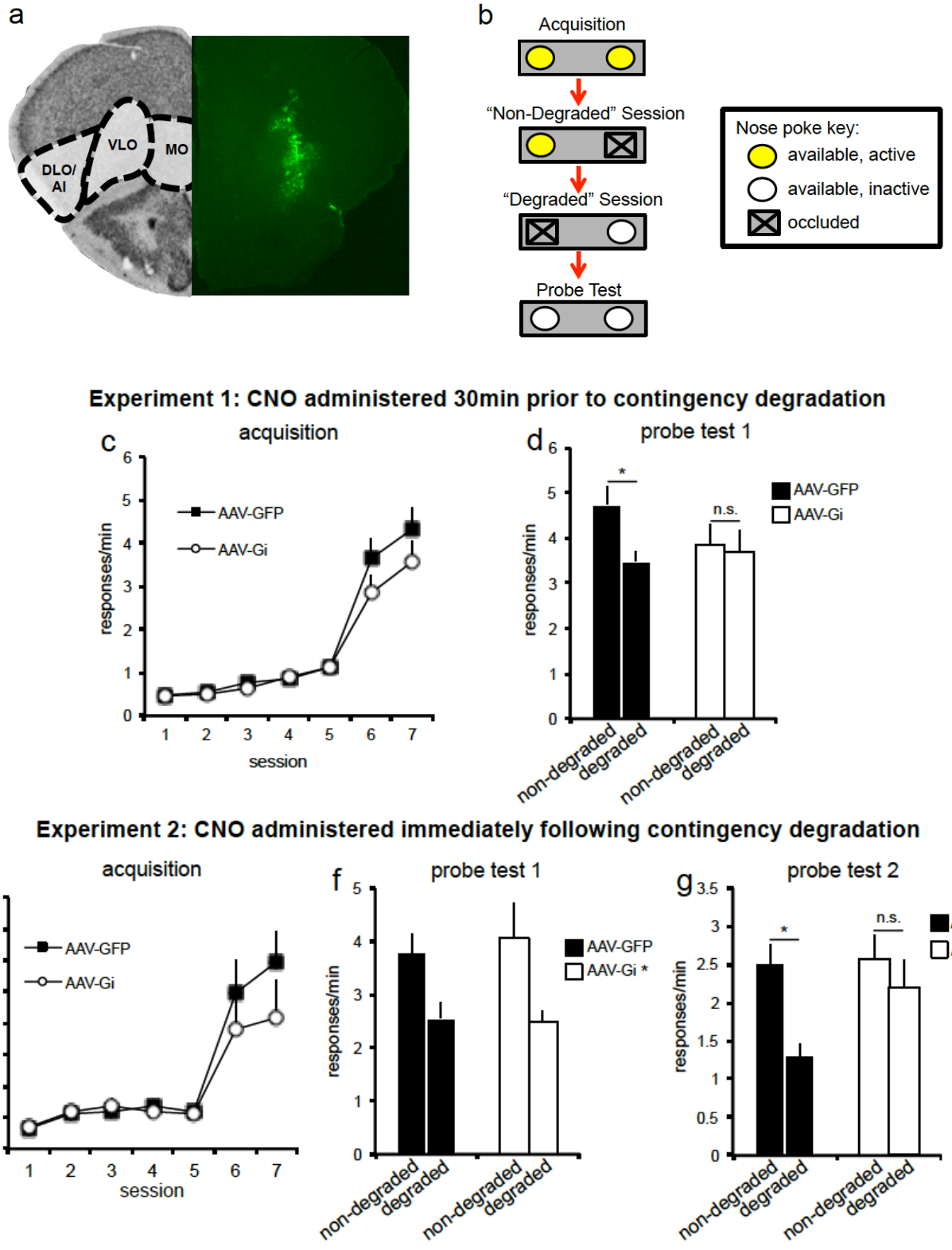
Finally, we used single-cell patch clamp recordings to resolve the mechanism by which Gi-DREADD activation was altering neuronal physiology. Surprisingly, we did not find changes in intrinsic membrane properties. This suggests that in our manipulations, Gi-DREADD activation did not hyperpolarize cells through β/γ subunit binding to GPCR inwardly-rectifying potassium (GIRK) channels, as has been previously described (Pei et al., 2008). In spite of this, Gi-DREADD-infected neurons exposed to CNO showed a significant increase in the threshold for LTP induction, which would point to a Gαi-mediated mechanism that inhibits adenylyl cyclase and its downstream effectors, cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). Indeed, it has been demonstrated that a postsynaptic PKA-dependent pathway is necessary for early LTP (<1 hour after HFS) in the hippocampus following a stimulation protocol very similar to the 5xHFS we applied (Blitzer et al., 1995). Therefore, the behavioral effects we report may be due to interference with early-phase LTP induction through downregulation of the postsynaptic PKA pathway, even in the absence of more immediate "silencing" effects via GIRK-mediated hyperpolarization. In addition to the effects we observed on early LTP, mobilization of the Giα subunit could also disrupt the later phases of LTP by inhibiting PKA-mediated gene transcription and protein synthesis (Mayr and Montminy, 2001; Sindreu et al., 2007).

Conclusions

Our findings indicate that the VLO is necessary for behavioral flexibility when new information changes the validity of a previously learned association. This influence is detectable irrespective of outcome valence, and can be attributed to effects on the consolidation of new

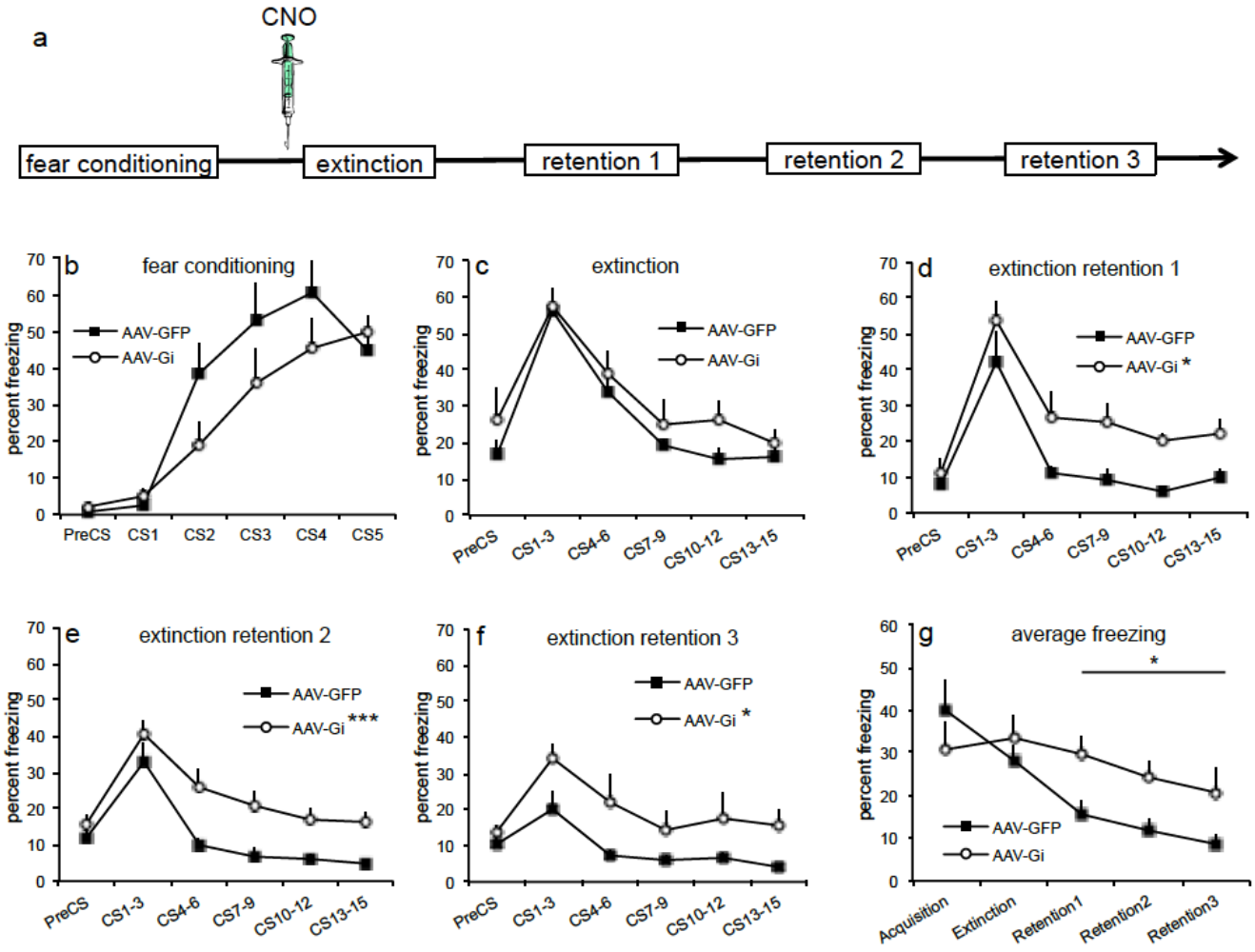
information regarding the predictive relationship between actions or stimuli and associated outcomes. Dysregulated VLO activity could be a factor in many disorders characterized by cognitive and behavioral inflexibility (see Introduction). Further characterizing the function of the VLO and understanding how it interacts with other prefrontal cortical and limbic regions to coordinate behavior will be an important step in studying the etiology and expression of these illnesses.

Figure 4-1: *DREADD-mediated inhibition of the VLO prevents stable consolidation of response-outcome contingency degradation.*



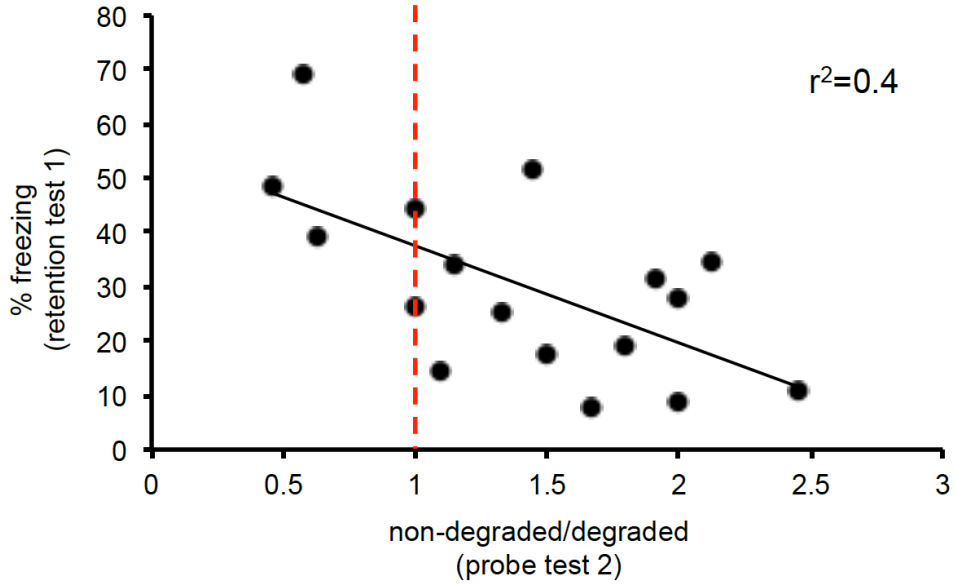
(a) Spread of the viral vector infusion was limited to the VLO. (b) A task schematic is shown. Mice are trained to generate two distinct responses. Then, the likelihood that one response will be reinforced is decreased. Preferential engagement of the remaining response during a probe test is interpreted as goal-directed action selection, while engaging both responses equivalently — despite response-outcome contingency degradation — is codified as habitual behavior. (c) Both GFP control and Gi-DREADD-infected mice successfully acquired the responses. Response acquisition curves represent both responses/min. (d) When CNO was administered prior to instrumental contingency degradation, the Gi-DREADD mice were unable to select between actions that were more, *vs.* less, likely to be reinforced (non-degraded *vs.* degraded) in the subsequent probe test; instead, they generated both responses equally, habitually. (e) A separate cohort of mice also successfully acquired the responses. (f) When CNO was injected immediately *following* instrumental contingency degradation, all mice initially generated a preference for the response that was most likely to be reinforced. (g) However, this preference decayed in Gi-DREADD-expressing mice in a second probe test, and their responding became non-selective, a habit-based response strategy. Symbols and bars represent means+SEMs, *$p<0.05$. Abbreviations: *DLO- dorsolateral orbitofrontal cortex; AI- agranular insula; VLO- ventrolateral orbitofrontal cortex; MO- medial orbitofrontal cortex.*

Figure 4-2: *DREADD-mediated inhibition of the VLO impairs the retention of fear extinction.*



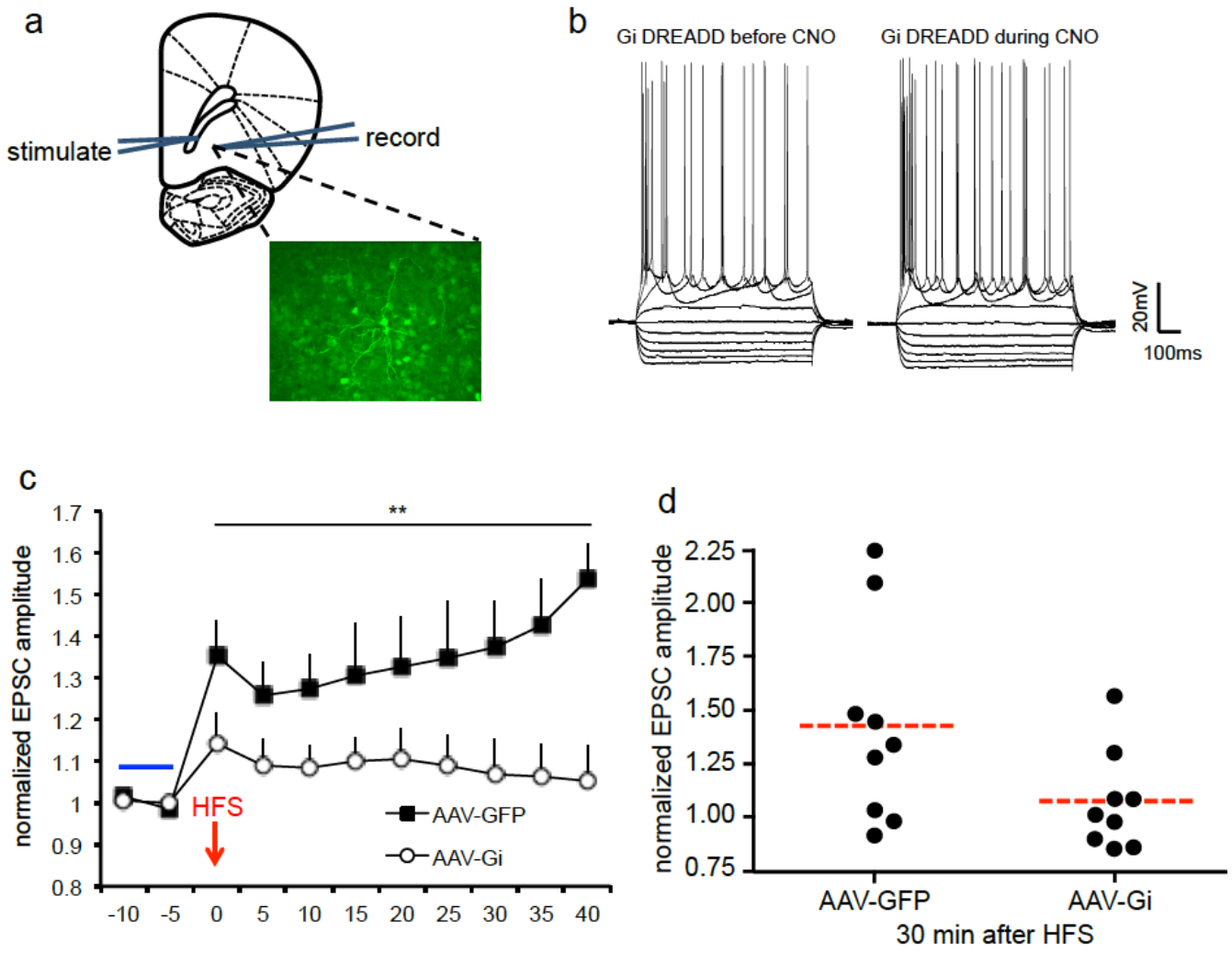
(a) Task timeline. (b) Freezing increased as a function of 5 CS-US pairings. (c) Freezing during extinction training (the presentation of 15 non-reinforced CSs) was not affected by CNO treatment. (d) During extinction retention testing, however, Gi-DREADD-expressing mice froze significantly more than control mice. (e-f) Increased freezing in the Gi-DREADD group compared to controls continued during two more days of unreinforced CS exposure. (g) Freezing across all sessions is shown, revealing a selective and long-lasting deficit in extinction retention in Gi-DREADD-expressing mice following a single injection of CNO. Symbols represent means+SEMs, *$p<0.05$, ***$p<0.001$.

Figure 4-3: *The expression of goal-directed decision-making strategies correlates with the retention of fear extinction.*



Mice that developed more pronounced goal-directed response strategies were more likely to show more successful fear extinction, characterized by lower levels of freezing during the first extinction retention test. The red dashed line indicates equal engagement of both responses. Each symbol represents a single mouse.

Figure 4-4. *Activation of Gi-DREADDs in the VLO increases the threshold for LTP induction.*



(a) The forceps minor of the corpus callosum was stimulated, and recordings were collected from nearby Gi-DREADD infected VLO neurons, identified by the expression of mCitrine (inset). (b) No apparent changes in intrinsic membrane properties during application of CNO (100µM) were detected. (c) Bath application of CNO prior to 5xHFS increased the threshold for LTP induction in principal VLO neurons infected with the Gi-DREADD compared to those infected with GFP. Time of CNO application is marked by the blue horizontal line. Symbols represent means+SEMs, **p<0.001. (d) Normalized EPSC amplitude for Gi-DREADD infected neurons

compared to GFP-infected neurons 30 minutes following HFS. Each symbol represents EPSC for one neuron.

Chapter 5:

Discussion

5.1 Summary of Results

The previous chapters describe how plasticity in two highly interconnected regions, the basolateral amygdala (BLA) and the orbitofrontal cortex (OFC), particularly the ventrolateral subregion (VLO), is necessary for associative conditioning and behavioral flexibility in appetitive- and aversive-based tasks. In Chapter 2, we determine the necessity of interactions between Brain-derived neurotrophic factor (BDNF) and tyrosine kinase receptor B (TrkB), its principal receptor, in the BLA for the formation of positively- and negatively-valenced stimulus-outcome associations, i.e., cocaine-conditioned place preference (CPP) and fear-potentiated startle (FPS). In Chapters 3 and 4, we discuss connectivity between the OFC and the amygdala, and describe how plasticity in the VLO facilitates the consolidation of new information about previously learned associations.

A previous study demonstrated that overexpression of a dominant negative isoform of TrkB impaired conditioned fear acquisition when selectively expressed in the BLA (Rattiner et al., 2004). In Chapter 2, we expand on these findings, first by demonstrating that viral-mediated knockdown of *Bdnf* in the BLA similarly impairs the acquisition of FPS – that is, mice with knockdown showed deficits in forming an association between a light conditioned stimulus (CS) and a shock, demonstrated by impaired FPS in the presence of the CS. This was not due to changes in baseline anxiety affecting fear behavior. We next showed that both *Bdnf* knockdown and overexpression of the dominant negative TrkB isoform in the BLA impaired the acquisition of cocaine-CPP, indicating that amygdalar production of BDNF as well as BDNF-TrkB interactions are necessary for the formation of both aversive and appetitive associative memories. Notably, once cocaine-CPP had been formed, TrkB inhibition in the BLA impaired

the extinction of the preference, suggesting that plasticity within the BLA is necessary for the flexible alteration of learned stimulus-outcome associations as well as their formation.

In Chapter 3, we first demonstrate patterns of direct connectivity between two regions of the OFC and the downstream striatum and amygdala. Furthermore, we show that bilateral *Bdnf* knockdown in the VLO, as well as a modified disconnection wherein *Bdnf* was knocked down unilaterally and the contralateral amygdala was lesioned, both caused inflexible, habitual response selection. This indicates that both BDNF-dependent plasticity within the VLO and VLO-amygdala connectivity are critical for goal-directed decision-making. In a previous study, *Bdnf* knockdown in the VLO resulted in decreased levels of the BDNF protein in the downstream striatum and amygdala (Gourley et al., 2013c). Here we found that following knockdown in the VLO, the expression of amygdalar BDNF correlated with goal-directed behaviors – that is, lower levels were predictive of habitual decision-making strategies. Together, this suggests a potential model in which anterograde BDNF transport from the VLO to the striatum and amygdala promotes plasticity at these sites and modifies learned associations. This would agree with data in Chapter 2 indicating that impairment of TrkB function in the BLA makes mice resistant to the extinction of cocaine-CPP. We developed our findings in Chapter 3 further by showing that systemic administration of a TrkB agonist or an agent that promotes remodeling of dendritic spines rescues flexible decision-making in mice that had been over-trained or had received VLO *Bdnf* knockdown. Finally, we also found that activation of a Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADD) in the VLO prevented the stable consolidation of new action-outcome conditioning, ultimately weakening goal-directed decision-making. Together, the findings in Chapter 3 suggest that BDNF-dependent plasticity in the VLO is necessary for the incorporation of new information about

previously acquired action-outcome associations, and that BDNF-dependent VLO-mediated promotion of behavioral flexibility is reliant on interactions with the amygdala.

In Chapter 4 we expand on our findings from Chapter 3 by understanding how DREADD-mediated VLO inhibition obstructs behavioral flexibility. We first replicate and expand upon our findings that Gi-DREADD activation during the consolidation of action-outcome contingency degradation destabilizes new learning, causing mice to rely instead on familiar, habitual response strategies. We next tested the effects of Gi-DREADD activation during fear extinction training and found that VLO inhibition prevented the consolidation of extinction conditioning, such that in subsequent tests these mice continued to freeze in response to the presentation of the CS, while control mice successfully extinguished CS-elicited freezing. **This suggests that the VLO is important for the modification of stimulus-outcome, as well as action-outcome, associations, and that it is recruited in tasks that require behavioral flexibility irrespective of the valence of the outcome.**

5.2 Integration of Findings with the Current Literature

Our data suggest a model of outcome-based associative learning and memory and behavioral flexibility that is dependent on local plasticity in the VLO and the BLA as well as, to some extent, functional connectivity between the two structures. However, in order to make informed conclusions about how these regions interact to mediate behavior, it is important to understand direct and indirect anatomical connectivity between them, as well as how other regions may interact with the network either synergistically or competitively. This section summarizes existing knowledge regarding the connectivity between subregions of the rodent PFC and amygdala, with some additional consideration of the striatum. Additionally, our results

will be integrated with a brief overview of existing literature discussing how prefrontal-amygdala networks regulate behavior, with an emphasis on Pavlovian fear conditioning and extinction as a model of associative learning and behavioral flexibility. Finally, we will discuss findings from human neuroimaging studies indicating that OFC structure and function are impacted in fear-related mood disorders. These findings contextualize our own work at a translational level.

The rodent PFC can be divided into subregions that constitute ***1)*** a medial portion, consisting of the anterior cingulate cortex (Cg1), the prelimbic cortex (PL), the infralimbic cortex (IL), and the medial OFC (MO), and ***2)*** a lateral portion, consisting of the ventral and lateral orbitofrontal cortices (here jointly referred to as the VLO), the dorsolateral OFC (DLO), and the agranular insular cortex (AI) (fig.5-1). These subregions all have connectivity with the amygdala and the striatum that is strongly topographically organized. For the purpose of the current discussion, Cg1, as well as the frontal association cortex and the motor cortices, will not be addressed. Moreover, although the amygdala is divided into multiple subnuclei, this chapter will primarily focus on projections targeting the BLA and the central nucleus (CeA), specifically the lateral/capsular division of the CeA (CLC).

5.2.1 Anatomical connectivity

The DLO and the AI

As demonstrated in Chapter 3, infusions of an anterograde tracer into the DLO/AI subregion in mice reveal massive innervation of the anterior BLA (fig.3-4c). Moving caudally, projections originating in the DLO terminate progressively more laterally and ventrally, ultimately appearing in the ventral BLA alone in the most posterior sections. The CeA is relatively devoid of terminals at all levels. This is consistent with previous reports in rats

(McDonald et al., 1996; Groenewegen et al., 1997), though notably, labeling in the BLA in these cases was considerably lighter than what we report. This could be due to the placement of infusions in these prior seminal reports using rats (Groenewegen et al., 1997; McDonald et al., 1996), which were in both cases slightly caudal to ours.

Moving in a rostral-caudal direction, the DLO transitions to the rostral AI approximately halfway through the PFC, when the claustrum first appears (Van De Werd and Uylings, 2008). The anterior AI is then divided into a dorsal AI (AID) and ventral AI (AIV), which are maintained until approximately the emergence of the bed nucleus of the stria terminalis, at which point the AID and the AIV become the posterior AI (AIP), which is no longer considered "prefrontal" (Van De Werd and Uylings, 2008). Notably, we find that infusions of an anterograde tracer into the AIP result in strong innervation of the CeA (particularly the lateral division) and comparative avoidance of the BLA (fig.5-2). Together, these patterns suggest parallel insular pathways composed of a prefrontal-BLA pathway and a nonprefrontal-CeA pathway. Furthermore, these findings highlight that comparatively denser innervation of the anterior BLA that we report in Chapter 3 compared to others may be due to the inclusion of the DLO and the rostral-most aspects of the AI in our infusion zone, compared to more selective posterior targeting of the AID reported by both (Groenewegen et al., 1997) and (McDonald et al., 1996).

The VLO

As described in Chapter 3, amygdala-targeting projections from the VLO were confined nearly exclusively to the BLA, with some light innervation of the ITC's also noted (fig.3-1d). Like the DLO/AI, terminals were densest in the anterior BLA, and in more caudal sections,

labeling became more laterally oriented. Again, this is consistent with previous descriptions in rats of projection patterns from the ventral and lateral OFC compartments (McDonald et al., 1996; Groenewegen et al., 1997). It must be noted however, that although projections to the BLA were consistent across multiple animals, in our findings and others' (McDonald et al., 1996; Groenewegen et al., 1997), labeling in the amygdala following VLO infusions is relatively light compared to other prefrontal regions. Therefore, it is important to consider other modes of connectivity between these two areas. As discussed in Chapter 3, VLO-BLA connections are known to be reciprocal, and previous studies in mice have demonstrated that the projections from the BLA to the VLO are quite pronounced (Matyas et al., 2014). Additionally, a thalamic relay forms an indirect pathway between the OFC and the amygdala (Timbie and Barbas, 2014). Our findings suggest that the VLO projects to the mediodorsal (MD) and the ventromedial (VM) thalamic nuclei (fig.5-3a). Projections *from* the MD *to* the BLA have been previously reported in rats (van Vulpen and Verwer, 1989); however a recent study in mice found no connectivity between the MD and the BLA, but showed that the VM thalamic nucleus was responsible for the majority of BLA innervation (Matyas et al., 2014) (see fig.5-3b). In either case, it appears that the VLO is ideally situated to mediate BLA activity indirectly through midline thalamic nuclei. Therefore, in addition to the direct pathway, the VLO may regulate BLA activity indirectly through the thalamus, while the BLA provides reciprocal feedback via a direct pathway to the VLO.

The MO

The MO subregion of the OFC is detectable in the rostral-most portion of the PFC, immediately medial to the VLO and ventral to the PL. Moving along the rostral-caudal gradient,

it remains ventral to the PL, and then to the IL in more caudal PFC sections. Projections from the MO to the amygdala have been reported throughout the BLA, though in more caudal sections, innervation becomes more medially oriented (Hoover and Vertes, 2011). Additionally, the same report demonstrates innervation of the CLC, although the medial subdivision of the CeA (referred to as the CeM) is relatively devoid of terminals. The complementary innervation of the lateral BLA by the DLO/AI and the VLO, and the medial BLA and the CLC by the MO, suggests that the OFC projects to the amygdala in a topographically-organized manner, and has the ability to differentially affect behavior through its distinct subregions.

The IL

The IL is situated directly above the caudal aspects of the MO and ventral to the PL, with which it is frequently compared and contrasted. Indeed, these two structures together are frequently referred to as the medial PFC (mPFC), or ventromedial PFC (vmPFC). Projections from the IL largely overlap with projections from the MO in the amygdala, though the IL terminal field is significantly denser in the CLC (McDonald et al., 1996). The IL also innervates the BLA, particularly in the medial aspects (Sesack et al., 1989; McDonald et al., 1996; Groenewegen et al., 1997). Notably, while projections from the orbitofrontal subregions (DLO/AI, VLO, and MO) largely target the basal aspects of the BLA, IL projections primarily appear more dorsally, in the ventral aspects of the lateral nucleus (Lv). This again reveals a topographical organization of prefrontal-amygdala inputs, wherein projections are not only oriented on a medial-lateral gradient, but also a dorsal-ventral gradient.

Some previous studies have demonstrated functional physiological connectivity between the IL and the ITCs, and this connectivity is widely thought to be behaviorally meaningful in the

context of fear conditioning and its extinction (Berretta et al., 2005; Li et al., 2011b). However, recent anatomical evidence suggests that direct connectivity is rare (Pinard et al., 2012), and innervation of the ITCs by the IL likely occurs via an indirect pathway through the BLA (Strobel et al., 2015).

The PL

The PL is a large subregion of the mPFC, spanning most of its rostral-caudal length. It is situated dorsally to the MO in rostral sections, and further caudally, it sits dorsally to the IL and ventrally to Cg1. PL projections to the amygdala largely overlap with the MO, and to some extent the IL, albeit with some important distinctions. PL innervation of the amygdala is most pronounced in the medial aspects of the basal nucleus, though moderate innervation of the Lv (where the IL preferentially terminates in the BLA) is also present (Sesack et al., 1989; McDonald et al., 1996; Groenewegen et al., 1997). Some innervation of the CLC is present, but PL innervation at this site is noticeably weaker than the IL. Notably, overlapping projections from the PL and the IL do not necessarily suggest similarities in function; the cell populations of amygdalar nuclei are highly heterogeneous, and preferential innervation of different subpopulations or inhibitory networks within the same site could affect behavior in dramatically different directions (Ciocchi et al., 2010; Haubensak et al., 2010; Wolff et al., 2014).

PFC Innervation of the Striatum

In addition to the amygdala, all PFC regions innervate the striatum, and these pathways must also be considered when attempting to understand dissociable functions of the separate PFC subregions. As demonstrated in Chapter 3, the VLO targets the central zone of the dorsal

striatum (DS) (fig.3-1b), while the DLO/AI sends projections more laterally and ventrally within the striatum (fig.3-3b). Within the PFC, it appears that the VLO has the weakest innervation of the ventral striatum (VS) and instead preferentially targets the dorsal aspects (Sesack et al., 1989; Groenewegen et al., 1997; Schilman et al., 2008). By contrast, the IL and the DLO/AI both strongly innervate the VS, particularly the ventral VS (below the anterior commissure), while the PL and the MO innervate the dorsal VS (Rodriguez-Romaguera et al., 2015).

5.2.2 Contributions to Fear Conditioning and its Extinction

As discussed above, the amygdala receives massive inputs from PFC regions, and in turn, the BLA sends significant reciprocal projections back to the PFC (McDonald, 1991; Hoover and Vertes, 2007). This substantial and highly organized anatomical relationship suggests that functional communication between these areas is both finely tuned and capable of mediating a variety of behaviors. The amygdala circuitry involved in Pavlovian (stimulus-outcome) fear conditioning is extensively studied and well-defined (Davis, 1992; Fanselow and LeDoux, 1999), and in recent years our understanding of how this process is mediated by mPFC structures has increased dramatically. However, comparatively little is known about the role of the OFC in fear conditioning and extinction in rodents. This section will integrate our behavioral findings from Chapter 4 into existing literature about PFC regulation of fear-related learning.

The dissociable roles of the IL and the PL in fear conditioning have been extensively studied – briefly, the PL facilitates the expression of fear responses following the acquisition of conditioned fear, while neural activity in the IL is necessary for extinction conditioning and the consequent suppression of fear responses (Sierra-Mercado et al., 2011). This is thought to be

largely mediated by the differential projection patterns of the PL and IL to the amygdala, as discussed in the previous section (Peters et al., 2009).

A recent study provided some of the first evidence generated using rodents that the OFC regulates fear conditioning. The authors demonstrated that inactivation of the MO inhibited fear expression during extinction training, but did not affect the expression of conditioned fear in subsequent tests (Rodriguez-Romaguera et al., 2015). The authors suggest that the MO regulates fear responses through its projections to the ventral VS, a site innervated by both the MO and the PL. In a previous study, the same group demonstrated that deep brain stimulation (DBS) of the *dorsal* VS facilitates fear extinction and induces phosphorylation of Extracellular-signal Regulated Kinase 1/2 (ERK1/2), a marker of synaptic plasticity, in regions associated with the "extinction network", for example the IL and the CLC (Rodriguez-Romaguera et al., 2012). Notably, this manipulation also increased phosphorylation of ERK1/2 in the VLO, which we demonstrated to be necessary for extinction conditioning in Chapter 4. Together, these findings suggest two parallel PFC networks – one "fear on" network composed of the PL and the MO, and one "fear off" network composed of the IL and the VLO (fig.5-4).

In dissecting what we know of the proposed "fear off" network, it seems conceivable that the VLO and the IL function synergistically to extinguish conditioned fear. First, activity in the IL could be sufficient to suppress freezing *during* extinction training, explaining the absence of an immediate effect of DREADD-mediated VLO inhibition, coupled with a DREADD-mediated impairment in the *retention* of extinction conditioning, in Chapter 4. In support of this model, muscimol-mediated inactivation of the IL during extinction training causes a pronounced blockade of within-session extinction (Sierra-Mercado et al., 2011), indicating that the IL is indeed involved in the rapid suppression of conditioned fear. This effect is maintained only

during extinction training (i.e., while the IL is inactivated) and in the early stages of a test session on the following day — by the end of the test session, both experimental and control animals equally extinguish the freezing response. It is important to note, however, that other manipulations of the IL (e.g., inhibiting gene transcription and translation) during extinction training do not affect the expression of fear *during* training, but disrupt extinction consolidation (Mueller et al., 2008), much like we report in Chapter 4. Therefore, it appears that both the IL and VLO must be functional for the successful consolidation and retention of extinction conditioning, while activity in the IL alone is sufficient to drive the initial rapid suppression of the fear response during extinction training.

The respective projection patterns from the VLO and the IL to the amygdala further suggest that each region could be facilitating extinction conditioning in a unique, and possibly synergistic, fashion. Specifically, the VLO preferentially innervates the BLA (Berendse et al., 1992; McDonald et al., 1996), which would allow it to update associations at the site where they are initially formed, for instance by weakening circuits that encode the fear memory or potentiating a competing extinction network within the BLA. In contrast, the IL sends strong projections to the lateral/capsular subdivision of the CeA (Berendse et al., 1992; McDonald et al., 1996), which contains a population of "fear off" neurons that suppress a complementary "fear on" population in the same area (Ciocchi et al., 2010; Haubensak et al., 2010). Therefore, the IL could act in a network complementary to the VLO by potentiating this extinction circuit in the CeA.

One remaining question concerns the role of the DLO/AI in fear conditioning and extinction. The projection patterns of this region overlap with the IL in the striatum and the VLO in the amygdala, suggesting that it is ideally situated to mediate fear extinction. However, to our

knowledge, relatively little about this area has been published related to Pavlovian fear conditioning. One report indicates that lesions of the DLO/AI delay the acquisition of contextual, but not cued, fear conditioning (Morgan and LeDoux, 1999). In this study, extinction was *not* affected, suggesting that the DLO/AI may instead be part of the "fear on" pathway. The insula is considered to be a site that processes interoceptive awareness of visceral states, including pain. Indeed, lesions of the rostral AI diminish sensitivity to inflammatory and neuropathic pain in rats (Coffeen et al., 2011). It is possible that the AI mediates learning associated with fear and reward by providing an interoceptive representation of the anticipated outcome, though further research is still needed to specifically address the contribution of this subregion to fear conditioning and extinction.

5.2.3 Translational Implications

The OFC is highly conserved across species, and in fact is considered by some to be the only region in rodents that can be qualified as classically "prefrontal" in the same way that the term is applied to primates (Preuss, 1995). In humans, abnormalities in OFC structure and function are thought to be involved in multiple psychopathologies. For instance, the OFC and the cingulate gyrus are two cortical structures commonly implicated in drug addiction in humans (Goldstein and Volkow, 2002); extensive rodent research has consequently delved into the mechanisms by which the OFC regulates reward-related decision-making (for example, see Chapter 3), and how these processes are affected by exposure to drugs of abuse (Stalnaker et al., 2009; Lucantonio et al., 2012).

Although rodent literature focusing on the OFC in the context of fear-based conditioning is minimal thus far, abundant evidence suggests that the human OFC is involved in disorders of

fear and anxiety (see (Milad and Rauch, 2007) for review). For instance, both decreased volume (Levy-Gigi et al., 2013; Sekiguchi et al., 2013) and hyperactivity (Huang et al., 2014) of the OFC are reported in individuals suffering from posttraumatic stress disorder (PTSD). Activity in the OFC also correlates with signs of physiological distress in patients with PTSD, indicating that the OFC has a role in top-down regulation of the stress response (Barkay et al., 2012). Additionally, regional blood flow to the OFC decreases when phobic subjects are presented with images of the feared stimuli (Fredrikson and Faria, 2013).

Of particular relevance to the findings we present in Chapter 4, decreased OFC volume has been repeatedly associated with failures in conditioned fear extinction (Milad et al., 2005; Levy-Gigi et al., 2013). Furthermore, in healthy subjects, negative prediction error — i.e., when an expected outcome is not delivered — is associated with OFC activity during fear conditioning (Spoormaker et al., 2011); negative prediction error is a critical step in successful extinction, and this evidence suggests that OFC activation could be necessary for it to occur. Together, these studies provide compelling evidence that the OFC is a significant regulator of fear and anxiety in both healthy and pathological contexts. Further investigation in rodents is an important next step in order to interpret these findings by elucidating the molecular, pharmacological, and physiological mechanisms by which this PFC region regulates aversive-based learning and memory. By defining the mechanisms through which stimulus-elicited fear can be regulated and successfully extinguished, novel treatment strategies for the treatment of fear- and anxiety-related mood disorders may be developed.

5.3 Implications and Future Directions

To summarize, the VLO is part of a PFC network that regulates associative conditioning and behavioral flexibility, at least in part via connectivity with the amygdala. We propose a model in which the VLO functions primarily by recognizing changes in the predictive relationship between an action or stimulus and its outcome, rather than by assessing the value or salience of the outcome itself. Furthermore, our findings reveal that plasticity within the VLO and the BLA is necessary for the formation and modification of both reward- and fear-based learning and memory. Frequently these opposing motivations are studied in isolation from one another; however, examining the neural correlates that are necessary for both positively- and negatively-valenced emotional learning is highly relevant from a translational perspective as we attempt to understand the risk factors and etiology of psychiatric illness. Although disorders like phobias, obsessive-compulsive disorder, and addiction vary widely in symptomatology, expression, and motivation, those suffering from them have in common the inability to disengage from destructive, habitual patterns of thought or behavior. Uncovering the neural correlates of such generalized risk factors as behavioral inflexibility allows for the possibility of pharmacotherapeutic solutions relevant to a broad spectrum of psychiatric disorders.

The findings presented in the previous chapters provide strong evidence that the VLO promotes behavioral flexibility via enhancing the consolidation of new learning, and one key experiment indicates that the VLO acts via interactions with the BLA. However, further research could more directly test the *necessity* and *sufficiency* of VLO-BLA interactions in facilitating goal-directed decision-making and other forms of behavioral flexibility. For instance, the importance of direct connectivity could be assessed by infusing a retrograde Cre Recombinase-expressing viral vector in the BLA and simultaneously infecting the VLO with a floxed DREADD virus. In this way, DREADDs are active specifically in VLO neurons that project to

the BLA. Replicating the behavioral experiments described in Chapter 4 would test the necessity of the direct VLO-BLA pathway in mediating behavioral flexibility in appetitive and aversive contexts. In the same fashion, projections *from* the BLA *to* the VLO could be manipulated to assess the influence of the reciprocal pathway.

Additionally of note, with the exception of the pharmacological interventions of Chapter 3, most of our manipulations were aimed at causing *impairments* in the behavioral tasks (deficits in the consolidation of new associations, resulting in behavioral inflexibility), demonstrating the necessity of the VLO and BLA in outcome-based learning and memory. Using Gs-DREADDs might afford us the opportunity to test *sufficiency*, for instance by activating the principal excitatory neurons of the VLO in over-trained mice in order to rescue goal-directed decision-making.

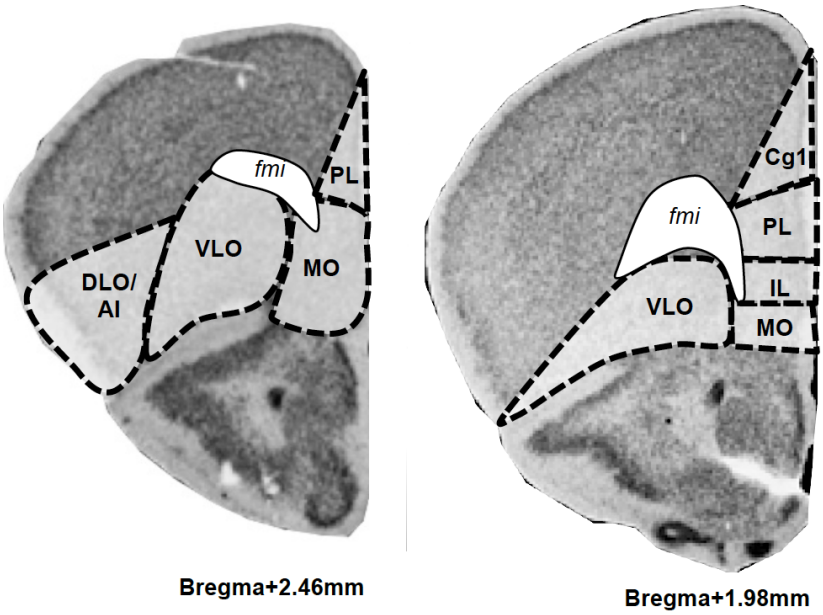
In addition to the VLO-BLA network, several other VLO projection sites stand out as potentially significant and novel targets for further research, particularly when attempting to define a role for the OFC in fear conditioning. For instance, the DS is well established as an important mediator of goal-directed decision-making, and we have previously demonstrated that disconnection of the VLO and the DS impairs goal-directed response selection (Gourley et al., 2013c). However, the contribution of the DS to fear conditioning is less studied. Lesions of this region impair cued fear conditioning, but not contextual fear conditioning (Ferreira et al., 2003); the same group later reported that this process is dependent on indirect connectivity with the CeA, possibly relayed through the substantia nigra pars compacta (SNc) (Ferreira et al., 2008). To our knowledge the role of the DS in fear extinction is not known. Considering the highly heterogeneous composition of the striatum, it is possible that one dorsal striatal pathway mediated by the CeA and the SNc promotes Pavlovian fear conditioning, and another, potentially

regulated by the VLO, facilitates extinction. Further functional and anatomical dissection of the VLO-DS pathway will be necessary to more comprehensively determine the mechanisms by which the VLO regulates behavioral flexibility in aversion-based tasks.

5.4 Conclusion

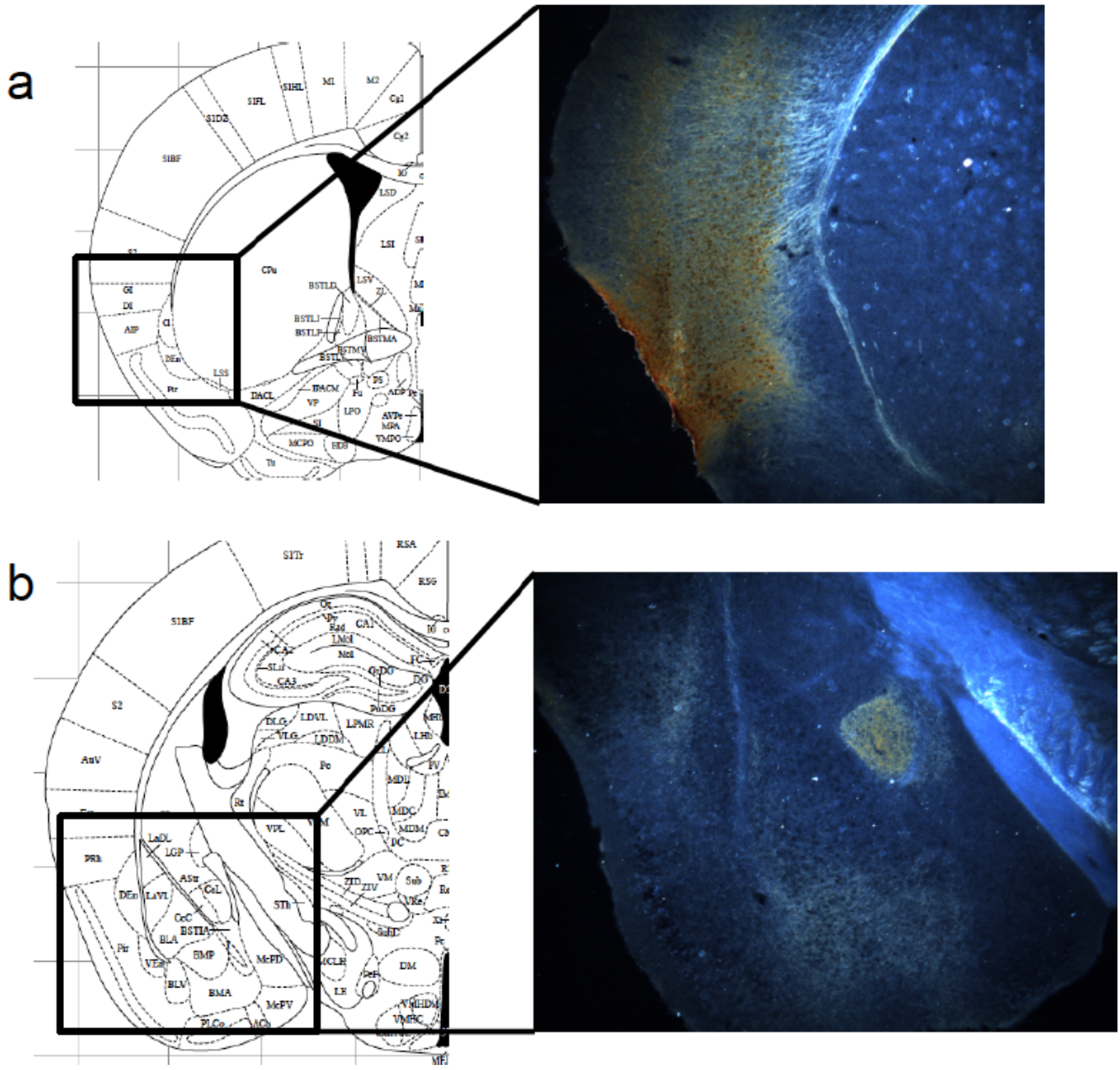
This thesis describes the manner in which plasticity in the BLA and VLO mediates associative conditioning and behavioral flexibility in both appetitive and aversive domains. These chapters contribute to a growing body of work that endeavors to understand the common mechanisms and pathways underlying reward- and fear-based learning and memory, with the ultimate goal of finding treatable targets for pathological states that are defined by cognitive and behavioral inflexibility.

Figure 5-1: *Subregions of the Rodent PFC*



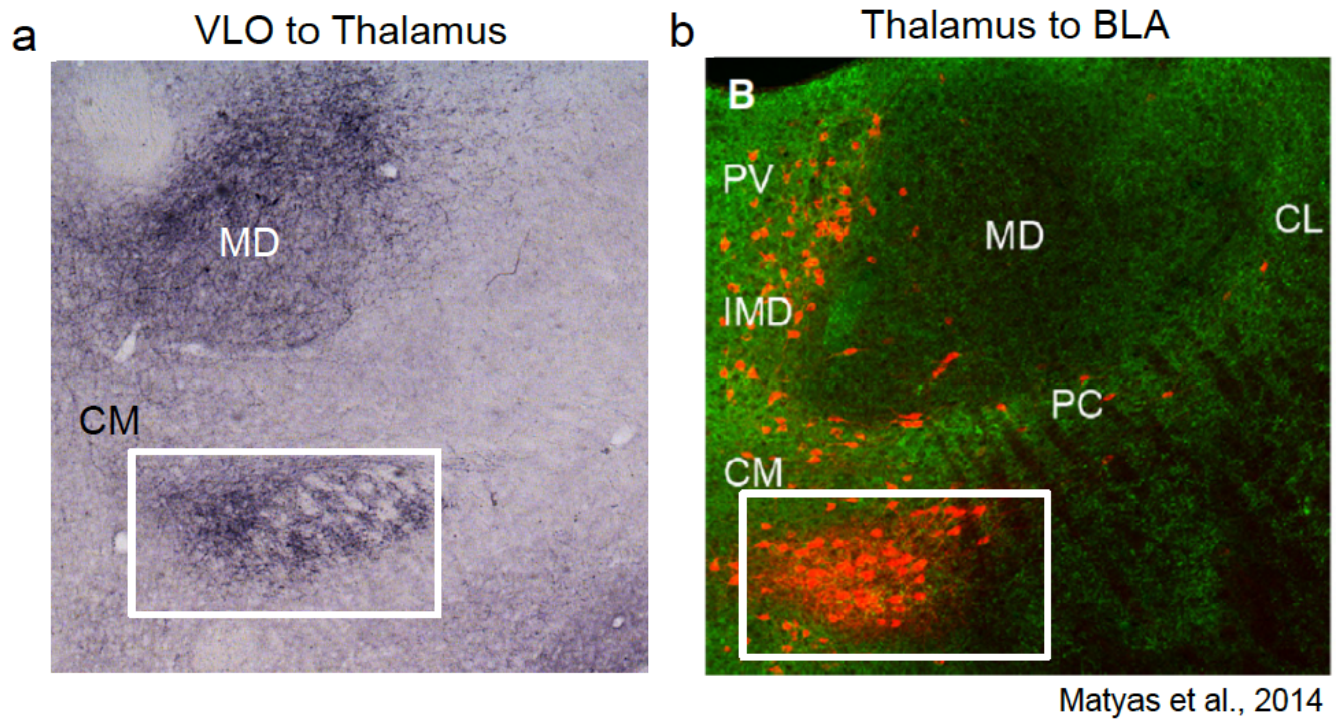
The PFC is divided into multiple subregions – the lateral PFC includes the dorsolateral OFC (DLO), the agranular insula (AI), and the ventrolateral orbitofrontal cortex (VLO), while the medial PFC includes the medial OFC (MO), the infralimbic cortex (IL), the prelimbic cortex (PL) and the rostral cingulate cortex (Cg1). "fmi" refers to the forceps minor of the corpus callosum. Regions are traced on images from the Mouse Brain Library.

Figure 5-2: *Posterior Insular Projections to the Amygdala*



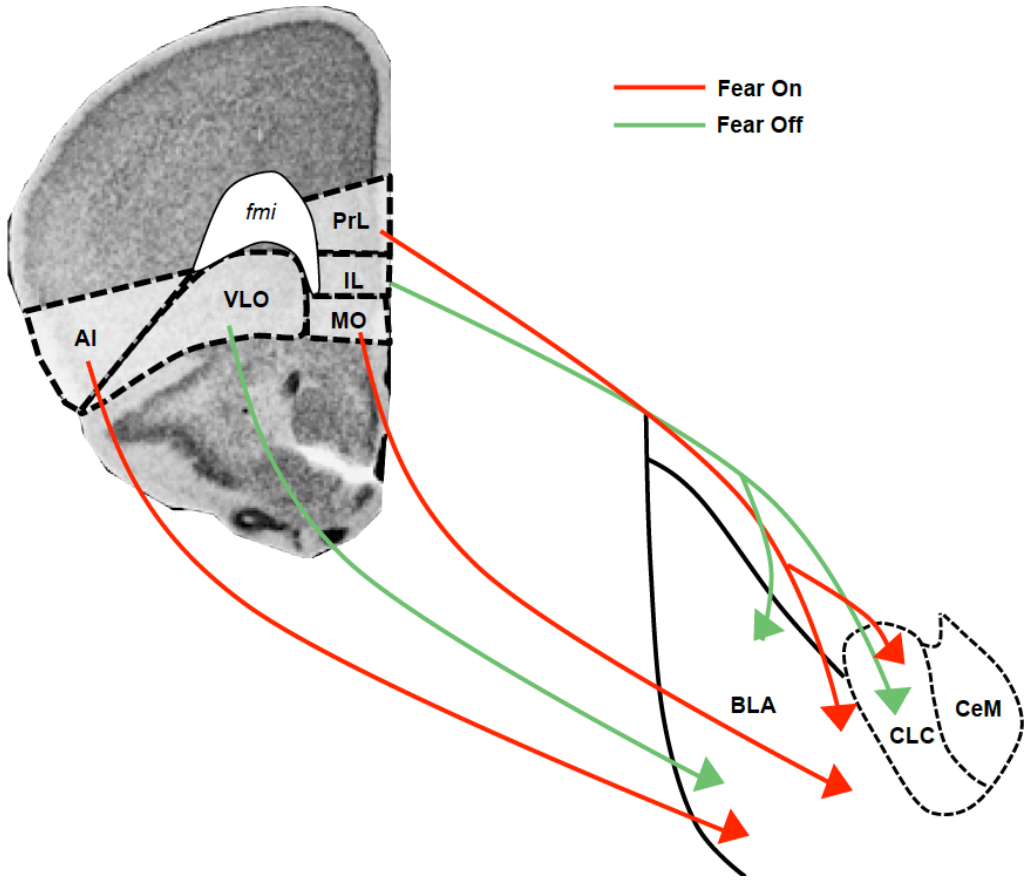
(a) BDA 10,000 was infused into the AIP. The diagram on the left from Paxinos and Franklin (2001) shows the region depicted in the image on the right (the infusion site). (b) The diagram on the left shows the region of the amygdala highlighted in the image on the right, which reveals strong innervation of the CeA by the AIP. Abbreviations: *AIP- posterior agranular insula; CeA- central nucleus of the amygdala.*

Figure 5-3: *Indirect Connectivity Between the VLO and the BLA*



(a) Innervation of the thalamus following an infusion of BDA 10,000 in the VLO is shown. (b) An image adapted from Matyas et al., 2014 shows labeled cells in the thalamus following an infusion of a retrograde tracer into the BLA. White outlines indicate the ventromedial thalamus, which is innervated by the VLO and projects to the BLA. Abbreviations: *MD- mediodorsal thalamus; CM- centromedial thalamus*; *PV- paraventricularis; IMD- intermediodorsal thalamus; PC- paracentral thalamus; CL- centrolateral thalamus.*

Figure 5-4: *Anatomical and Functional PFC-Amygdala Connectivity*



Subregions of the PFC project in topographically distinct patterns to the amygdala. Red lines indicate pathways that purportedly promote fear expression ("fear on"), while green lines indicate pathways thought to promote fear extinction ("fear off"). Abbreviations: *AI- agranular insula; VLO- ventrolateral orbitofrontal cortex; MO- medial orbitofrontal cortex; IL- infralimbic cortex, PL- prelimbic cortex; BLA- basolateral amygdala; CLC- lateral/capsular amygdalar nucleus; CeM- centromedial nucleus; fmi- forceps minor of the corpus callosum.*

Appendix A: Publications to which the Author has Contributed

- **K.S. Zimmermann**, K.J. Ressler (2010) Inhibition of Protein Kinase G Facilitates the Consolidation of Fear Extinction. *Emory University Undergraduate Honors Thesis.*
- S.L. Gourley, A. Olevska, **K.S. Zimmermann**, K.J. Ressler, R.J. Dileone , J.R. Taylor (2013) The orbitofrontal cortex regulates outcome-based decision-making via the lateral striatum. *Eur J Neurosci.* 38(3):2382-8.
- S.A. Heldt*, **K.S. Zimmermann***, K. Parker, M. Gaval, D. Weinshenker, K.J. Ressler (2014) Bdnf Deletion or TrkB Impairment in Amygdala Inhibits Both Appetitive and Aversive Learning. *J Neurosci.* 34(7):2444-50.
  *Contributed equally to the paper
- L.M. DePoy, R.E. Perszyk, **K.S. Zimmermann**, A.J. Koleske, S.L. Gourley (2014) Adolescent cocaine exposure simplifies orbitofrontal cortical dendritic arbors. *Frontiers in Pharmacology.* 5:228.
- **K.S. Zimmermann**, J. Yamin, D.G. Rainnie, K.J. Ressler, S.L. Gourley (in revision) Connections of the mouse orbitofrontal cortex and regulation of action selection by BDNF-TrkB.
- **K.S. Zimmermann**, C.C. Hsu, J.R. Taylor, S.L. Gourley (in prep.) Strain differences in acute sensitivity to instrumental contingency degradation in mice.
- **K.S. Zimmermann**, C. Li, D.G. Rainnie, K.J. Ressler, S.L. Gourley (in prep.) Orbitofrontal cortical regulation of behavioral flexibility in appetitive and aversive domains.
- S.L. Gourley*, **K.S. Zimmermann***, J.R. Taylor (in revision) BDNF in the medial orbitofrontal cortex regulates sensitivity to outcome value.
  *Contributed equally to the paper
- L.M. DePoy, **K.S. Zimmermann**, S.L. Gourley (in prep.) Incubation and reversal of adolescent cocaine-induced habits.
- G.M. Gafford, K. McCullough, **K.S. Zimmermann**, R. Andero, K.J. Ressler (in prep.) Support for a phosphodiesterase-mediated mechanism in the amygdala during consolidation of fear extinction.

Chapter 2 References

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