Investigating the influence of LH-projecting BLA neurons upon motivated behavioral responding and appetitive learning

by

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A.B., Neurobiology
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Abstract

To optimize survival, organisms must be able to learn contingencies between external stimuli and rewards and appropriately respond to these associations. Deficits in reward-related learning or reward-seeking are thought to occur in a host of psychopathologies, including depression (Drevets, 2001), eating disorders (Wagner et al., 2007), and substance abuse (Wrase et al., 2007), such that improved understanding of reward processing could potentially aid in the development of therapies. Two neural regions, the basolateral amygdala (BLA) and lateral hypothalamus (LH), are both implicated in reward processing (Adamantidis et al., 2007; Anand and Brobeck, 1951; Brobeck, 1946; Gutierrez et al., 2013; Hoebel and Teitelbaum, 1962; Kempadoo et al., 2013; Margules and Olds, 1962; Muramoto et al., 1993; Sakurai, 2007; Schoenbaum et al., 1998; Tye and Janak, 2007; Tye et al., 2008, 2010), but the role of the BLA’s projection to LH in appetitive conditioning and reward-seeking remains unclear. Through the use of optogenetic techniques in mice, I have investigated the influence of LH-projecting BLA neurons upon motivated behavioral responding, which has indicated that the projection may support intracranial self-stimulation (ICSS). Further experiments with in vivo extracellular electrophysiological recordings from LH-projecting BLA neurons may also shed light on the encoding properties of these neurons during appetitive learning.

Thesis Supervisor: Kay Tye
Title: Assistant Professor Neuroscience
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Introduction

In order to survive, an organism must be able to learn and appropriately respond to environmental contingencies between stimuli and rewards. Perturbations to reward processing are thought to contribute to numerous psychopathologies, including depression (Drevets, 2001), eating disorders (Wagner et al., 2007), and addiction (Wrase et al., 2007), such that enhanced understanding of the biological mechanisms of reward processing could have tremendous therapeutic benefit. The basolateral amygdala (BLA) has been shown to play a critical role in the association of environmental cues and appetitive outcomes (Muramoto et al., 1993; Schoenbaum et al., 1998; Tye and Janak, 2007; Tye et al., 2008, 2010) as well as the ability of these cues to gain motivating properties (Tye et al., 2010). Despite these findings, the functions of the BLA’s efferent connection to the lateral hypothalamus (LH) in reward processing are not entirely known, as pre-existing techniques such as electrophysiology and electrical stimulation did not permit targeting of specific projections. Through the use of optogenetic techniques, I have investigated the contribution of the projection from the BLA to LH to motivated behavioral responding, in order to shed light on this connection’s possible role in appetitive learning and reward-seeking behavior.

Implication of BLA in Appetitive Learning

The BLA is a cortical-like structure of predominantly glutamatergic projection neurons that includes three nuclei: the lateral amygdala (LA), basolateral amygdala (BL), and basomedial amygdala (BMA) (Pape and Pare, 2010). Although substantial work has focused on the BLA and fear conditioning (LeDoux et al., 1990; Maren and Quirk, 2004; Pape and Pare, 2010; Quirk et al., 1995, 1997; Wilensky et al., 1999), the region has also been implicated in the association of cues, or conditioned stimuli (CS), and appetitive outcomes, or unconditioned stimuli (US). Electrophysiological recordings in rats and non-human primates (NHP) have revealed a subset of
BLA neurons that respond to reward-predictive cues (Muramoto et al., 1993; Sanghera et al., 1979; Tye and Janak, 2007; Uwano et al., 1995), and a subpopulation of BLA neurons is thought to represent the value of reward-predictive cues (Baxter and Murray, 2002; Belova et al., 2008; Paton et al., 2006; Schoenbaum et al., 2007). In NHPs, these neurons show a graded response depending on the magnitude of the reward, as well as activity that tracks reward-predictive cues following any changes in cue-outcome pairings (Belova et al., 2008; Paton et al., 2006). Another subpopulation exhibits a similar pattern of activity, but in response to aversive outcomes, indicating both positive and negative coding cells in the BLA.

In addition to representing value, the BLA may also endow a reward-predictive cue with reinforcing properties (Baxter and Murray, 2002). In the extinction phase of an operant appetitive conditioning task, rats will continue to nosepoke to trigger a cue, even after they discontinue checking for an appetitive outcome (Tye and Janak, 2007). This persistent nosepoking, long after the cue ceases to predict an outcome, indicates that the cue has acquired reinforcing properties. The firing of a subset of BLA neurons correlates with this behavior, indicating the BLA’s involvement in supporting reinforcement.

Consistent with this implication, BLA lesions have also produced deficits in 2\textsuperscript{nd}-order conditioning and reinforcer devaluation (Hatfield et al., 1996; Málková et al., 1997). In 2\textsuperscript{nd}-order conditioning, a CS\textsubscript{1} previously paired with a US is able to act as a reinforcer, causing an animal to respond to a CS\textsubscript{2} paired with CS\textsubscript{1}, even in the absence of the original US. BLA lesions impair 2\textsuperscript{nd}-order, but not 1\textsuperscript{st}-order, conditioning, indicating that the region contributes to the ability of the CS to act as a reinforcer. In reinforcer devaluation, an animal learns that a CS predicts a US (food), and the US is later devalued through pairing of the food with malaise, in the absence of the CS. BLA lesions also impair reinforcer devaluation, suggesting that the BLA is involved in updating the value of the CS. Thus, the BLA is involved in motivated behavioral responding, via
its possible endowment of cues with reinforcing properties and signaling of incentive value of the cue.

As found with fear conditioning (Maren, 2005; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005), cellular and synaptic changes consistent with long term potentiation occur alongside cue-reward learning in the BLA. After an appetitive operant (Tye et al., 2008) or Pavlovian auditory conditioning task (Namburi et al., 2015), a subset of BLA neurons exhibited potentiation of putative thalamic synapses, reflected by an increase in the AMPA/NMDA ratio, which is thought to indicate glutamatergic synaptic strength. This potentiation is considered NMDA-dependent, given that infusion of an NMDA-receptor antagonist prior to training diminished reward-learning performance (Tye et al., 2008). Blockade of NMDA-receptors also reduced the amplitude of mini excitatory postsynaptic currents (mEPSCs) in ex vivo slice recordings following training, as compared to animals receiving vehicle alone, which is consistent with decreased AMPA receptor number or function. Other studies have similarly reported impaired acquisition of Pavlovian appetitive tasks with infusion of an NMDA-receptor antagonist into the BLA (Burns et al., 1994). However, lesion studies of the BLA have not reported any deficits in 1st-order Pavlovian appetitive conditioning (Hatfield et al., 1996; Holland et al., 2001), although adaptation or redundant circuitry may account for this discrepancy.

Given the overlap in inputs that show potentiation in response to appetitive and aversive conditioning, the valence of a given BLA neuron may arise from its projection target (Namburi et al., 2015). Indeed, BLA neurons that project to the nucleus accumbens (NAc), a region implicated in reward-related processes (Caine et al., 1995; Hurd et al., 1989; Pettit and Justice Jr., 1989), show bidirectional modulation of potentiation depending on the type of conditioning, with an increase in the AMPA/NMDA ratio after appetitive learning, and a decrease after
aversive learning (Namburi et al., 2015). Inverse changes in AMPA/NMDA ratio are observed in BLA neurons projecting to the central medial nucleus of amygdala, which is heavily implicated in aversive conditioning (Ciocchi et al., 2010; Haubensak et al., 2010; Jimenez and Maren, 2009). Thus, investigation of the BLA according to projection target can yield important insight into the region’s contribution to associative learning, yet the role of the BLA’s connection to LH, another region implicated in reward, remains unclear.

Role of the LH in reward-related processes

The LH is a large and remarkably heterogenous region that has been implicated in reward, motivation, and consummatory behaviors (Adamantidis et al., 2007; Anand and Brobeck, 1951; Brobeck, 1946; Gutierrez et al., 2011; Harris et al., 2005; Hoebel and Teitelbaum, 1962; Kempadoo et al., 2013; Margules and Olds, 1962; Sakurai, 2007). Early studies of LH demonstrated intracranial self stimulation (ICSS) with electrical stimulation of LH (Olds and Milner, 1954), as well as grooming and sexual behaviors (Singh et al., 1996), and extracellular recordings revealed neurons responsive to reward-associated cues (Mora et al., 1976; Schwartzbaum, 1988). Further work has shown populations of LH neurons that encode reward predictive cues, reward retrieval, and unexpected omission of reward (Nieh et al., 2015).

The projection of the LH to ventral tegmental area (VTA), a crucial region in reward learning (Schultz, 2006; Schultz et al., 1997), has been found to contribute to reward-related behaviors. Electrical (Bielajew and Shizgal, 1986) and optogenetic (Kempadoo et al., 2013) activation of this projection supports ICSS, and these neurons appear to encode the learned action of pursuing an appetitive outcome, even if the outcome is unavailable (Nieh et al., 2015). LH neurons responsive to reward-predictive cues and unexpected omission of reward appear to be downstream of VTA neurons that receive input from LH, and they may receive information from the VTA either directly via a reciprocal connection or indirectly, via the nucleus.
accumbens, hippocampus, ventral pallidum, and amygdala (Barone et al., 1981; Beckstead et al., 1993; Simon et al., 1979). Thus, the BLA could potentially convey information from the VTA and/or thalamic inputs among others, necessitating further study of the BLA to LH projection.

Identification of the BLA to LH projection

The connection between BLA and LH was identified through histological and viral techniques. The projection was first labelled via an anterograde tracer, Phaseolus vulgaris leucoagglutinin (PHAL), in rats, which showed an efferent connection to LH from posterior BLA and anterior BMA, as well as adjacent nuclei, including CEA, anterior amygdaloid and cortical amygdaloid nuclei. Through the use of biotinylated dextranamine and immunostaining for melanin concentrating (MCH), BMA/CoA neurons were found to project directly onto MCH+ neurons in the LH (Niu et al., 2012). Researchers also demonstrated that the BLA, BMA, and BMP project either directly or indirectly onto orexin neurons in LH by expressing a retrograde tracer, a tetanus toxin fused to GFP, in orexin neurons. However, the tracer’s ability to jump more than one synapse precludes definitive determination of monosynaptic projection (Sakurai et al., 2005). Another study employing a pseudorabies virus with Cre-dependence also indicated that BLA neurons may project to leptin & neuropeptide-Y containing neurons (DeFalco et al., 2001), although, again, the virus employed could travel more than one synapse retrogradely. While more precise methods are needed to determine the monosynaptic connections of LH-projecting BLA neurons, these studies suggest the potential influence of the BLA upon neuropeptides crucial to energy homeostasis and arousal (Berthoud and Münzberg, 2011). Given that appetitive learning often pertains to unconditioned stimuli that affect energy, this projection from BLA to LH, as well as the reciprocal connection from LH to BLA, may provide an important link in the valuing of stimuli in relation to homeostasis.
LH-projecting BLA neurons respond to appetitive conditioned stimuli

Although, as discussed, extensive work has implicated the BLA and LH in reward-related processes (Adamantidis et al., 2007; Anand and Brobeck, 1951; Brobeck, 1946; Gutierrez et al., 2011; Hoebel and Teitelbaum, 1962; Kempadoo et al., 2013; Margules and Olds, 1962; Muramoto et al., 1993; Sakurai, 2007; Schoenbaum et al., 1998; Tye and Janak, 2007; Tye et al., 2008, 2010), the efferent connection between BLA and LH has only been investigated through lesion and histological studies (Holland et al., 2002; Petrovich and Gallagher, 2003; Petrovich et al., 2002, 2005). The projection is thought to contribute to CS-potentiated feeding (Holland et al., 2002; Petrovich et al., 2002, 2005), a phenomenon in which a CS will trigger eating in sated animals who previously ignored food, provided the animal learned the association of CS and food in a previous food-restricted state (Zamble, 1973). The assay may capture processes that contribute to overeating and obesity in humans (Petrovich and Gallagher, 2003; Rodin, 1981). Bilateral lesions of the BLA, but not the central amygdala (CEA), abolishes CS-potentiated feeding, without affecting acquisition of the CS-US pairing or baseline food consumption (Holland et al., 2002). Contralateral, but not ipsilateral, lesions of the BLA and LH similarly impair CS-potentiated feeding (Petrovich et al., 2002). Through the use of a retrograde tracer and staining for immediate early genes, researchers also reported activation of BMA/BLA neurons that project to LH in response to the CS+ in CS-potentiated feeding, far more than another cue that was paired to no outcome (CS−) (Petrovich et al., 2005). This indicates that LH-projecting BLA neurons may evoke the value of the CS (Petrovich and Gallagher, 2003), potentially as it relates to energy homeostasis.

Contribution of LH-projecting BLA neurons to BLA functions remains unknown

Beyond these studies of CS-potentiated feeding, little is known about the projection’s contribution to other functions attributed to the BLA. BLA and LH contralateral lesions do not...
impair 2\textsuperscript{nd}-order conditioning, but more temporally precise manipulations could reveal an effect, similar to that found with the projection from BLA to NAc (Setlow et al., 2002). Different BLA projections have been found to promote or diminish anxiety-related behaviors, with BLA to ventral hippocampus exhibiting an anxiogenic influence (Felix-Ortiz et al., 2013) and BLA to CeA an anxiolytic effect (Tye et al., 2011). However, any effect of LH-projecting BLA neurons upon anxiety-related behaviors remains unclear. The projection from BLA to NAc supports ICSS (Stuber et al., 2011), yet the effect of LH-projecting BLA neurons upon this process also has not been studied. Given the role of both BLA and LH in reward-related processes, investigation of this projection in the context of reward-seeking and appetitive learning is warranted, as well as control studies of anxiety-related behaviors.

\textit{Investigating the role of LH-projecting BLA neurons in positive reinforcement}

Through optogenetic techniques, I have investigated the projection from BLA to LH in a temporally and spatially specific manner, in order to identify the influence of these neurons upon motivated behavioral responding, as well as support future studies of this projection’s role in appetitive associative learning. I have found preliminary evidence that BLA to LH supports ICSS, without pronounced effects upon locomotion or anxiety-related behaviors. Given that activation of LH neurons supports ICSS, it is likely that more precise experiments will verify these results, but further controls are required to substantiate my findings. I have also demonstrated that identification of LH-projecting BLA neurons in \textit{in vivo} extracellular recordings is possible, and that such recordings could be conducted during acquisition of an appetitive conditioning task in order to study the encoding properties of these neurons.
Methods

All procedures were performed according to the guidelines from the NIH and with approval of the MIT IACUC and DCM.

Animals and Stereotaxic Surgery

For all stereotaxic surgeries, adult wildtype male C57BL/6 mice (8.3 ± 1.5 weeks; Jackson Laboratory, Bar Harbor, ME) were used. All surgeries were performed under aseptic conditions with a digital small animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA), and all mice were anaesthetized with isoflurane (5% for induction, 1.5-2.0% after) during surgery. A heating pad was used to maintain body temperature throughout surgery, and afterwards, animals were subjected to a heating lamp until fully recovered. Following recovery, animals were housed in a 12 hour reverse light-dark cubicle (darkness from 9 am to 9 pm) to promote wakefulness during behavioral assays.

Viral transduction of BLA neurons and optic fiber implantation over LH

In order to manipulate the activity of LH-projecting BLA neurons with optical stimulation, wildtype mice underwent stereotaxic surgery with viral infusion in the BLA and optic fiber implantation over the LH (Fig. 1A). I infused the BLA unilaterally with 0.425 μl of adeno-associated virus (AAVs) carrying the opsin fused to a fluorophore, hChR2(H134R)-eYFP, under a Ca²⁺/calmodulin-dependent protein kinase IIα (CaMKIIα) promoter at stereotaxic coordinates from bregma: -1.5 mm anteroposterior (AP), +3.35 mm mediolateral (ML) and -4.89 mm dorsoventral (DV). This virus has been shown to produce expression of ChR2 in glutamatergic BLA neurons (Tye et al., 2011). In a second group of animals, I infused a fluorophore control virus (AAV5-CaMKIIα-eYFP), and surgery upon ChR2 and eYFP animals was interleaved throughout the day. For each group of animals, I used a different NanoFil syringe (10 μL; WPI, Sarasota, FL) with a beveled 33 gauge needle and infused the virus at a
Figure 1. Stereotaxic surgery for optogenetic manipulations and recordings. 
A, BLA neurons are transduced with ChR2-eYFP, and BLA terminals in LH are activated during behavior via stimulation with blue light (473 nm). B, LH-projecting BLA neurons are transduced with ChR2-eYFP through infusion of a retrograde cre-recombinase virus in LH and a cre-dependent virus carrying ChR2-eYFP in the BLA. An optic fiber is implanted over the BLA to activate LH-projecting BLA neurons during behavior. C, LH-projecting BLA neurons are transduced with ChR2-eYFP using the same method as B, followed by optrode implantation in the BLA for extracellular recordings during behavior.
rate of .1 µL /min using a microsyringe pump (UMP3; WPI, Sarasota, FL) and controller (Micro4; WPI, Sarasota, FL). After infusion, the syringe was raised 50 µm and left for twenty minutes to allow diffusion of virus and guard against leakage of virus along the needle tract.

After viral infusion, a 300 µm optic fiber (0.37 numerical aperture, NA) glued to a 2.5 mm stainless steel ferrule (ThorLabs) was implanted over the LH (-.5 mm AP, +1.05 mm ML, AP -.5, 4.85 mm DV). The pre-implantation efficiency of all fibers was greater than 80%. Adhesive cement (C&B metabond; Parkell, Edgewood, NY) was applied to the skull and fiber, followed by cranioplastic cement (Dental cement; Stoelting, Wood Dale, IL) to anchor the implant. Twenty minutes following cement application, the incision was closed with nylon sutures. Based on optogenetic studies with the same virus in our lab, animals were given 4-5 weeks post-surgery with ad libitum food and water to insure sufficient opsin in BLA terminals during behavioral assays. Surgeries for two of these animals, one of which was excluded due to viral leak, was performed by a labmate, Chris Leppl.

Specific viral transduction of LH-projecting BLA neurons

In order to control for stimulation of fibers of passage, as well as perform "phototagging" during in vivo extracellular recordings, another group of animals underwent stereotaxic surgery to transduce only LH-projecting BLA neurons with opsin. Using the same stereotaxic techniques described above, I injected 1 µl of a retrograde virus, canine adenovirus 2 (CAV2) carrying cre-recombinase, into the LH ( -.5 mm, + 1.1 mm ML, -5.1 mm DV) (Fig. 1B). In some animals, I injected a mixture (1:1) of CAV2-cre and AAV5-CaMKIIα–mCherry to allow visualization of injection site, given that CAV2-cre lacks a fluorophore. In the BLA, I infused an AAV5 containing a ‘double-floxed’ inverted open reading frame (DIO) and hChR2(H134R)-eYFP under a nonspecific neuronal promotor, the GTP-binding elongation factory family, EF-1α. A second group of animals received an infusion of virus with a control fluorophore (AAV5-EF1α-
DIO-eYFP). Given that only BLA neurons projecting to LH contain cre-recombinase with this method, only the BLA to LH projection express ChR2-eYFP or control fluorophore, assuming accurate infusion of virus in both regions. This method is referred to as “cre-DIO” hereafter.

With the same technique described previously, I implanted an optic fiber over the BLA to permit optical stimulation of these neurons during behavioral assays. Animals were subjected to behavioral assays 3-6 weeks following surgery, given the need for troubleshooting regarding optimal timepoints to run animals with this technique.

Implantation of Chronic Optrode for In Vivo Electrophysiological Recordings

A subset of cre-DIO animals was reserved for in vivo extracellular electrophysiological recordings and did not receive an optic fiber implantation following viral infusion. These animals were maintained in the animal facility for 6-10 weeks following viral infusion, prior to implantation of a chronic optrode, an electrode with an optic fiber (construction described below), via a second surgery (Fig. 1C).

During the second surgery, a craniotomy was drilled over the BLA for the optrode (-1.4 mm AP, +3.4 mm ML), and another smaller craniotomy over the posterior ipsilateral hemisphere for the ground wire. Four skull screws were implanted around the optrode craniotomy. The optrode was attached to a blue laser (473 nm, 20 mW) and the head-stage of the RZ5 recording system (TuckerDavis Technologies, Alachua, FL, USA) to permit recording of electrical activity while driving the optrode stereotaxically through the brain. The ground wire was secured at a depth of ~1.5 mm in the second craniotomy, after the optrode was lowered to +1.5 mm DV. The optrode was then driven down at approximately 0.01 mm/s until a depth of +4.7 mm DV, before delivering optical stimulation (10 Hz, 5 ms pulse for 1 second(s)) to detect photoresponsive units, in which units show time-locked firing in response to illumination. If such firing was observed, constant illumination (1 s, every 30 s) was delivered to rule out a photoelectric effect,
in which electrical activity increases at light onset and offset, but does not represent a photoresponsive unit. If the unit showed firing during the 1 s illumination (with possible tapering due to blockade of sodium channels), the optrode was secured at that depth. If no photoresponsive units appeared with optical stimulation, the optrode was lowered another 0.05 mm and illumination repeated. If no photoresponsive units were identified after reaching a maximal depth of +5.15 mm DV, the optrode was retracted and re-implanted +.2mm AP from previous coordinates. The same process was repeated and if no photoresponsive units were found, the optrode was cemented (-1.6 mm AP, +3.4 mm ML, +5 mm DV) to avoid further damage due to electrode tracks. The same cementing technique described previously was performed, although more layers of cement were added and more time allotted between layers to insure immobility of the implant. After the cement fully dried, the animal was disconnected from the headstage and laser, and the incision was sutured. The animal was allowed to recover for 1-2 weeks with ad libitum food and water before behavioral assays and recordings began.

In vivo optogenetic manipulation

Virus construction and packaging

The recombinant AAV vectors were serotyped with AAV5 coat proteins and packaged by the University of North Carolina Vector Core (Chapel Hill, NC). The maps of these constructs are available online at www.optogenetics.org. The CAV2-cre virus was produced by Montpelier Vectorology at BioCampus Montpelier (Montpelier, France).

Light delivery

During optogenetic behavioral assays, optical stimulation was delivered from a 100 mW 473 nm DPSS laser (OEM Laser Systems, Draper, UT). The laser was connected to a fiber-optic rotary joint via a patch cord with FC/PC connectors on both ends (OEM Laser Systems, Draper, UT), which was linked to another patch cord with a FC/PC connector on one end and a ferrule
with diameter matching the animal’s implant on the other end. The mouse’s implant was attached to the patch cord via a ceramic mating sleeve (PFP, Milpitas, CA), and the rotary joint allowed the animal to freely move without perturbation of optical stimulation. Light delivery was modulated with a Master 8 pulse stimulator (A.M.P.I., Jerusalem, Israel) and triggered by behavioral hardware (MedPC Associates, St. Albans, VT).

During in vivo extracellular recordings, the patch cord from the laser was attached to a mechanical/optical commutator (Tucker-Davis Technologies), and light delivery was controlled by the recording software (Tucker-Davis Technologies).

Behavioral assays

Animal Habituation to Handling

Two days prior to behavioral assays, I habituated animals to experimenter handling. In each session, one per day for two days, cages were transported from the animal facility to the laboratory behavioral rooms, and each animal was handled for 5 minutes. Handling consisted of scruffing each animal, attaching the animal to a patch cord, allowing the animal to explore while attached to the patch cord, and disconnecting the animal from the patch cord. After handling, the animals were housed in darkness in the lab behavioral rooms for an hour before returning the cages to the animal facility. An undergraduate, Melodi Anahtar, assisted with habituation of animals.

Intracranial self-stimulation (ICSS)

4-5 weeks following surgery, animals were food restricted overnight and underwent ICSS, an assay in which each animal was subjected to a self-stimulation session on two consecutive days. For each session, animals were attached to a patch cord and allowed to freely explore a dark, sound-proof conditioning chamber (MedPC Associates, St. Albans, VT) equipped with auditory-stimulus generators and an infrared video camera, as well as an active
and inactive nosepoke (Fig. 2A). Each session was initiated with the onset of low volume white noise to mask any ambient noises. The active nosepoke triggered a tone and 1 second of optical stimulation (473-nm, 20 Hz, 5-ms, 20 mW), and the inactive nosepoke triggered 1 second of a distinct tone and no stimulation. Tones (1 and 1.5 kHz) and location of nosepokes were counterbalanced across all animals. On the first day, both nosepokes were baited equally with food (Fruit Loop), and, on the second day, the nosepokes were not baited. Access to food (4 hours) was given upon completion of the first day’s session, and each animal was run at approximately the same time each day. The apparatus was thoroughly cleaned with an acetic acid solution between all animals to avoid bias, which an undergraduate, Melodi Anahtar, provided assisted with. Med-PC software recorded timestamps for each nosepoke, and performance on the second day was assessed using MATLAB and Microsoft Excel.

*Open field test (OFT)*

To assess effects of optogenetic manipulation upon locomotion and anxiety-related behaviors, each animal underwent an OFT assay. Each animal was attached to a patch cable and allowed to recover for 1-5 minutes prior to placement in an open field apparatus, a transparent plexiglass box (50 x53 cm) (Fig. 2B). Within the software, the apparatus was divided into zones: the center region (25 x 25 cm) and a border region (periphery). The 15-minute session consisted of five alternating 3-minute epochs (OFF-ON-OFF-ON-OFF) with optical stimulation during ON epochs (20 Hz, 5 ms, 10 mW). Laser power was reduced as compared to the ICSS assay due to the longer duration of stimulation. During the behavioral assay, the experimenter remained hidden behind an opaque screen, and the assay was conducted within a dimly lit room. A video camera overhead recorded the animal’s behavior, and an EthoVision XT video tracking system (Noldus, Wageningen, Netherlands) acquired the mouse’s location, velocity, and movement of
Figure 2. Behavioral assays.

A, In ICSS, animals are placed in a behavioral chamber that includes an active nosepoke, paired to a tone and optical stimulation, and an inactive nosepoke, paired to a second tone and no optical stimulation. Optical stimulation consists of a 1 s train of 20 Hz, 5 ms pulses (20 mW, 473 nm). B, Animals explore an open field apparatus during 3-min alternating epochs of optical stimulation with 20 Hz, 5 ms pulses (10 mW, 473 nm) and no stimulation. C, Animals are exposed to a food pellet and novel object for 3-min on two consecutive days, with optical stimulation with 20 Hz, 5 ms pulses (10 mW, 473 nm) occurring on one of these days. D, Naive wildtype mice are exposed to a pair of novel objects to insure no object preferences. E, The apparatus for the partial reinforcement operant conditioning task consists of a nosepoke and sucrose port, with houselight atop the sucrose port.
head, body, and tail. Melodi Anahtar helped compile the data from the epochs in Ethovision XT. Microsoft Excel was used to further analyze this data.

Novelty & Feeding Assay

Animals were subjected to a novelty & feeding assay to determine effects of optical stimulation upon novel object exploration and feeding behaviors. The novelty & feeding assay consisted of two sessions, one per day, on consecutive days (Fig. 2C). Animals were attached to a patch cable and allowed to recover for 1-5 minutes before placing the animal in its home cage (all cagemates and the cage’s nesting material was placed in another cage during the assay). The home cage, as well as habituation, was used to reduce exploration of the environment and promote interaction with the object and food, in accordance with previous findings regarding object exploration and familiarity of environment (Besheer and Bevins, 2000). During each session, the animal was allowed to habituate for 1 minute in the cage, followed by a 3 minute exploration epoch. During the exploration epoch, a novel object and food pellet were placed at opposite ends of the cage, away from the cage wall. Optical stimulation (20 Hz, 5 ms pulses, 10 mW) was delivered during the exploration epoch of one day’s session and omitted during the other day’s session. The order of stimulation, order of objects, and location of objects were counterbalanced across all animals, particularly as novel object assays can show an order effect, with increased exploration on the first day as compared to the second day. Novel objects (plastic princess figurine, constructed Lego figure) were previously tested with a separate group of animals, who exhibited no object preferences (described below). Novel objects were cleaned with an ethanol solution between each animal and handled with new gloves. Food pellets, identical to those normally used to feed the animals, were obtained from the animal facility, and a new pellet, handled with new gloves, was used for each animal. For each animal, new gloves
were not allowed to contact any surface, object, or food item other than the given novel object or food pellet prior to placement of the object in the cage.

Each session was recorded with a video camera overhead and analyzed by an observer using ODLog behavioral analysis software (Macropod Inc., USA) to quantify object and food interaction, digging, and eating behaviors. Object and food interaction (duration) was scored such that proximity of the head within less than 2 cm of the object, in the direction of the object, qualified as interaction. Rearing upon the object, or sitting near the object with the head directed away, did not qualify as interaction, consistent with previously published methods (Antunes and Biala, 2011).

Object Preference Assay

Object preferences among animals can create bias in novel object assays. While individual preferences in experimental animals cannot be tested (the object would no longer be novel in subsequent assays), the behavior of a separate group of wildtype mice can be used to control for this possibility. Wildtype mice (n = 10) were placed in an enclosure, and two novel objects (princess figurine, constructed Lego figurine) were deposited at either end, away from the walls (Fig. 2D). The mice were allowed to freely explore for 10 minutes, and the video recording was scored using ODLog software for interaction with each object, with the same criteria described previously. The location of the objects was counterbalanced across all animals.

Partial Reinforcement Sucrose Self-Administration Task

For in vivo extracellular recordings, an animal with an implanted optrode underwent an appetitive operant conditioning task, in which each behavioral session was followed by a "phototagging" session. Animals were food restricted for several days prior to the first training session, and then attached to a headstage and patch cable (as described in previous sections). The animal was placed in a dark, sound-proofed conditioning chamber (Med-PC Associates, Durban,
VT) which contained an auditory stimulus generator, infrared video camera, houselight, nosepoke, and sucrose delivery port (30% sucrose solution in cage water) (Fig. 2E). Low volume white noise was played throughout all sessions to mask ambient noise. In the first training session, the nosepoke was baited (Food Loop), and the animal was allowed to freely explore the apparatus. 50% of nosepokes triggered a 30-second tone (1 kHz), illumination of the houselight (2.45 s) and delivery of a small volume of sucrose to the port (~.73 ml). If the animal did not collect the sucrose, subsequent nosepokes triggered the tone without further sucrose delivery, until the animal visited the port. For other nosepokes, no tone, houselight illumination, or sucrose delivery was triggered, to control for motor activity in neural responses. The behavioral session concluded once the animal collected 100 trials worth of sucrose, or an hour and a half had passed. While still recording, the task was ended and a “phototagging” session was conducted to identify any units as photoresponsive.

**Visualizing viral transduction and fiber optic placement**

**Histology**

Due to difficulties with seizures while the optimal timepoint for behavioral assays was determined, animals did not undergo a c-fos stimulation protocol. Instead, mice were anesthetized with pentobarbital sodium and transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in PBS (pH 7.3). Brains were extracted and fixed in 4% PFA overnight and then transferred to 30% sucrose in PBS to equilibrate for 2-4 days. A sliding microtome (HM430; Thermo Fisher Scientific, Waltham, MA) was used to collect 40 μm-thick coronal sections, which were stored in PBS at 4°C. When immunohistochemistry was conducted, sections were washed in Triton 0.3%/PBS and 3% normal donkey serum for one hour, followed by incubation of sections with a DNA specific fluorescent probe (DAPI : 4',6-Diamidino-2-Phenyldimole (1:50,000)) for 1 hour with Triton 0.1%/PBS and 3% normal donkey serum at
room temperature. Sections were then washed 4 times with PBS-1X and mounted on microscope slides with PVD-DABCO. Melodi Anahtar assisted with a subset of these perfusions and some sectioning of these brains.

Confocal microscopy

Confocal fluorescence images were collected using a 10X/0.40NA or a 40X/1.30NA oil immersion objective on an Olympus FV1000 confocal laser scanning microscope. Serial Z-stack images were collected using the image analysis software (Fluoview, Olympus, Center Valley, PA). Mice exhibiting eYFP somata expression in the central amygdala, putamen, or piriform cortex were excluded from analysis (n=7).

Statistical analysis

Statistical analyses were conducted using Microsoft Excel and Matlab software. The threshold for significance for all results was $p = .05$ (denoted with *, $p < .01$ with **). All group data are displayed as the mean ± standard error of the mean (sem). Single variable differences within within subject comparisons were found with two-tailed paired Student t-tests. Group differences with two variables were analyzed with two-way ANOVA with Bonferroni post-hoc tests.

In vivo extracellular electrophysiology

Optrode Construction

I constructed optrodes by attaching a 300 μm fiber (.37 NA) within a stainless steel ferrule (1.25 mm diameter) to a 16-channel multielectrode array (Innovative Neurophysiology, Durham, NC, USA). The ferrule was cemented to the electrode so that the optic fiber tip formed an approximate 10 degree angle with the electrode tips, at a distance of 500 to 1500 μm. The optrode was constructed with this angle to insure that the light cone illuminated the electrode tips.
Behavioral Learning Curves

To assess learning within the task, I employed a state space model that utilizes the expectation maximization algorithm to estimate an individual animal’s learning curve (the probability of a correct response at each trial) (Smith et al., 2004). This state-space model paradigm estimates learning at each trial, which enables precise comparisons between behavioral changes and firing activity. In this paradigm, the learning criterion is defined by the confidence limits of the learning curve; the learning trial occurs when the lower bound of the 90% confidence interval remains above the probability expected by chance (50%) for the remainder of the session. Analysis began by identifying each trial (initiated by a nosepoke) as correct (1) or incorrect (0), with a correct response constituting sucrose retrieval within 10-s of tone onset, or refraining from sucrose retrieval in the absence of a tone. A learning curve was generated from this binary series by adapting MATLAB scripts, written by Anne Smith and obtained from the Brown lab at MIT.

In Vivo Electrophysiological Recordings and Phototagging with ChR2

Following each day’s behavioral session, phototagging with 473 nm laser (30-40 mW) commenced within the same recording session, in order to identify photoresponsive units. The phototagging session consisted of pseudorandomly dispersed optical stimulation of 1 s constant light or 10 s of 1 Hz light (5 ms pulses), with 10 repetitions of each stimulation type.

Electrophysiological data and behavioral timestamps were exported from the TDT system. Plexon offline sorter was used to sort waveforms with principal component analysis, and further analysis was conducted with NeuroExplorer and MATLAB.

Visualizing optrode placement

Prior to sacrificing an animal implanted with an optrode, electrolytic lesions were created to localize electrode tips. After anesthesization with isoflurane (5 % for induction, 1.5-2.0 %
after), a 19.6 mA current (15 s) was passed through each channel with sorted units. After sufficient time for gliosis passed (30 minutes), the animals were anesthetized with pentobarbital and perfused with the same technique described previously.
Results

Optical stimulation of BLA fibers in LH supports ICSS

To test whether optogenetic activation of BLA terminals in LH supports ICSS, I expressed Channelrhodopsin-2 (ChR2)-eYFP fusion protein in BLA pyramidal neurons in experimental animals and eYFP in control animals matched for age, incubation time, and illumination parameters. Prior to the behavioral assay, I implanted an optic fiber over the LH, in the hemisphere ipsilateral to the viral infusion. Following all behaviors, confocal imaging of tissue sections revealed viral transduction of BLA neurons without leakage into adjacent areas and correct placement of the optic fiber (ChR2, n = 8; eYFP, n = 7) (Fig. 3). The data from all animals that showed viral leakage or incorrect fiber placement were excluded from primary analysis (n = 7). The surgeries for two ChR2 animals, one of which was excluded due to viral leak, were performed by Chris Leppla.

ChR2 animals that underwent the ICSS assay showed a robust preference for the active over the inactive nosepoke during the second, unbaited session (Fig. 4A). The difference in nosepokes between active and inactive nosepokes among ChR2 animals was statistically significant as compared to eYFP (p < .01) (Fig. 4B). A representative trace showing a ChR2 animal’s performance over the course of a session is shown (Fig. 4C). Of note, animals that showed viral leak into CEA did not exhibit a preference for either nosepoke, despite ample expression in the BLA.

ICSS most likely arises from reward-related processes

To determine the effects of optical stimulation upon locomotion or anxiety-related behaviors, I conducted an OFT assay, which consisted of five alternating 3-minute epochs of optical stimulation (OFF-ON-OFF-ON-OFF). A subset of ChR2 animals (n = 3) were excluded from analysis due to seizures during this assay, even with minimal illumination, and were not
Figure 3. Confocal imaging reveals viral transduction of BLA and fiber placement over LH. 
A, A confocal image shows ChR2-eYFP (green) expression in the BLA. B, A confocal image displays optic fiber placement (red) over the LH with fibers expressing ChR2-eYFP (green). C, A confocal image from another animal shows a magnified view of correct fiber placement over the LH. D, An example of viral leakage beyond the BLA, into the CEA, which resulted in exclusion of this animal from primary analysis.
Figure 4. Optical stimulation of BLA fibers supports ICSS.
A, ChR2 animals (blue, n = 8) exhibited a robust preference for the active over inactive nosepoke during the second, unbaited session of ICSS, as compared to eYFP controls (red, n = 7). B, ChR2 animals’ preference for the active nosepoke, as reflected by the mean difference in active and inactive nosepokes, was highly statistically significant as compared to eYFP controls (p < .01). C, A representative trace displaying a ChR2 animal’s performance in the second, unbaited session of ICSS, with active nosepokes (blue) increasing over the course of the session.
subjected to any further assays. Only eYFP controls run during the same session as non-seizing ChR2 animals were retained in further analysis. Of the remaining ChR2 animals (n = 5), there was no substantial change in locomotion as compared to eYFP controls, reflected by similar mean distances moved and velocities across epochs (Fig 5A,B). ChR2 animals also exhibited similar anxiety-related behaviors to eYFP controls, with similar mean duration in the center and border zones of the apparatus (Fig. 6A). For the second epoch of optical stimulation, ChR2 animals exhibited an increase in time spent in the center zone, but this difference was not significant. A representative ChR2 animal’s movements within the apparatus’ zones are shown (Fig. 6B).

Novel object exploration & feeding behavior in a subset of ChR2 animals

Given the implication of the BLA to LH projection in CS-potentiated feeding, the remaining ChR2 animals (n = 2) were subjected to a novelty & feeding assay. A novel object was included in this assay to reveal any effects upon exploration and anxiety-related behaviors. ChR2 animals displayed similar levels of novel object exploration with and without optical stimulation (Fig. 7A), as well as similar exploration of food with and without optical stimulation (Fig. 7B). ChR2 animals showed a lower interaction difference as compared to eYFPs, with the interaction difference calculated by subtracting the duration of interaction with the food pellet from interaction with the novel object (Fig. 7C). Experimental animals displayed no substantial differences in digging (Fig. 7D), and optical stimulation did not elicit any feeding behavior in either experimental or control animals. As a control, wildtype animals were subjected to a separate assay to test for object preferences, but there was no difference in interaction time with each of the novel objects (Fig. 8A).

Absence of optical self-stimulation with putative transduction of LH-projecting BLA neurons

The ICSS observed with viral transduction of BLA neurons may arise from stimulation of
Figure 5. ICSS in ChR2 animals does not arise from a locomotor effect.
A, ChR2 animals (blue, n = 5) showed no difference in mean distances moved (± sem, cm) during ON and OFF epochs, or as compared to eYFP controls (red, n = 5). B, ChR2 animals exhibited no changes in mean velocity (± sem, cm/s) across epochs, or as compared to eYFP controls. ON epochs delivered 20 Hz, 5 ms pulses (10 mW, 473 nm).
Figure 6. Anxiety-related behaviors do not appear to contribute to ICSS in ChR2 animals. 
A, ChR2 animals (blue, n = 5) did not show a significant change in mean duration (± sem) spent in the center zone as compared to eYFP controls (red, n = 5). B, A representative track of a ChR2 animal’s movement during OFF (black) and ON (blue) epochs through border and center zones (dashed line).
Figure 7. Preliminary results of novel object exploration and feeding with optical stimulation. 
A, ChR2 animals (blue) did not show a significant difference in mean novel object interaction (± sem, s) or food interaction (B), as compared to eYFP animals (red) with optical stimulation of 20 Hz, 5 ms pulses (10 mW, 473 nm). C, The interaction difference, reflecting the difference between novel object and food interaction, was not significantly different with optical stimulation among ChR2 animals, although lower than with eYFP controls. D, ChR2 animals displayed no substantial change in time spent digging with optical stimulation or no stimulation relative to eYFP animals.
Figure 8. Absence of object preference among naive wildtype animals. In a control assay to rule out object preferences among animals, wildtype mice (n = 10) showed no significant difference in interaction time (± sem, s) with either novel object used in the novelty & feeding assay.
fibers of passage, rather than terminals in LH, particularly since the BLA possesses efferent connections to the ventromedial hypothalamus (VMH) (Petrovich et al., 2001). To control for this possibility, I used a cre-DIO method to selectively transduce LH-projecting BLA neurons with ChR2 and implanted an optic fiber over the BLA ipsilateral to viral injections. These animals underwent the ICSS assay at four and five weeks following surgery, but did not exhibit a preference for the active over inactive nosepoke at either timepoints (Fig. 9A). They also showed no difference in locomotion or anxiety-related behaviors as compared to eYFP controls (Fig. 9B). However, histological confirmation of viral transduction and fiber placement was not conducted.

*Feasibility of in vivo electrophysiological recordings during operant appetitive conditioning*

While optical self-stimulation observed with CamKIIα-ChR2 animals may indicate that this projection supports motivated behavioral responding, the actual encoding properties of these neurons in the context of appetitive learning remains unclear. In order to characterize the firing activity of LH-projecting BLA neurons, I selectively transduced these neurons with ChR2 using the cre-DIO method in order to permit phototagging of units during electrophysiological recordings (n = 2). During optrode implantation, I observed short latency (< 8ms), time-locked firing in response to optical stimulation (1 s constant & 10 Hz, 5 ms pulses) in one animal (Fig. 10A). After behavior, confocal imaging confirmed viral transduction of BLA neurons and electrode localization in the BLA (Fig. 10B). An example of sorted units, while not photoresponsive, from a behavioral session with the partial reinforcement operant conditioning task is shown (Fig. 10C).

Although photoresponsive units were not present during subsequent recording sessions, the animal was able to acquire an operant appetitive conditioning task while attached to the headstage and patch cable, which can sometimes interfere with the animals’ ability to perform. A
Figure 9. No significant effect of optical stimulation of putative LH-projecting BLA neurons. 
A, ChR2 (blue) and eYFP (red) animals did not show a preference for the active over inactive 
nosepoke during ICSS. B, The mean difference in active and inactive nosepokes (±, sem) was not 
statistically significant among ChR2 (n = 6) animals, as compared to eYFP controls (n = 2). C, 
ChR2 animals (blue) exhibited no substantial change in mean distance moved (± sem, cm) across 
epochs or in comparison to eYFP controls (red). D, ChR2 animals showed no substantial differ-
ence in mean time spent in the center zone (± sem, s).
Figure 10. Successful transduction of LH-projecting BLA neurons with ChR2-eYFP and in vivo extracellular recordings. A, During optrode implantation, a unit shows short latency (<8ms), time-locked firing in response to 1 s constant photostimulation (20 mW, 473 nm) and 10 Hz photostimulation for 1 s (5 ms pulses). B, A confocal image shows an electrolytic lesion on the border of the BLA, with viral expression of ChR2-eYFP (green) limited to the BLA. C, Representative clusters show cell sorting through principal component analysis and corresponding waveforms (D), reflecting neural activity recorded during the animal’s acquisition of the task.
learning curve from the animal’s second recording session displays the learning trial (Fig. 11). This curve was generated using code that I adapted from scripts written by Anne Smith, which are accessible through the Brown Lab website. Previously published electrophysiological recordings with this task in mice had only been conducted after the animal learned the task, which precluded analysis of acquisition (Nieh et al., 2015).
Figure 11. Behavioral learning curve displays animal’s acquisition of the task.
The learning curve (red) shows an animal’s performance on the second session of a partial reinforcement operant conditioning task, with incorrect trials (dark grey) in the beginning and correct trials (light grey) increasing over the session. The learning trial (green arrows) occurs when the lower bound of the 90% CI (blue lines) remains above the probability expected by chance (black line) for the rest of the session.
Discussion

*Projection from BLA to LH may support motivated behavioral responding*

Through optogenetic techniques, I have shown that the projection from BLA to LH may support optical self-stimulation, which has not been previously investigated. Previous methods did not permit selective, temporally precise manipulation of LH-projecting BLA neurons, as most studies have relied upon lesions, which allow for adaptation and effects upon fibers of passage. With viral transduction of BLA neurons and a fiber over the LH, ChR2 animals demonstrated a strong preference for the active over inactive nosepoke.

While the animals’ optical self-stimulation indicates a possible role for this projection in motivated behavioral responding, this result could also reflect non-reward related phenomenon. For example, an effect of optical stimulation upon locomotion or behavioral flexibility could prevent the animal from moving away from the active nosepoke. However, a subset of ChR2 animals did not display any difference in locomotion, as compared to eYFP controls, in the OFT assay. Optical stimulation could also prevent the animal from switching its current strategy, either by diminishing exploration or promoting perseveration. However, no difference in center zone exploration appeared during the OFT assay in experimental animals as compared to controls. Although ChR2 animals exhibited a lower baseline of novel objection exploration than eYFPs, in the novelty & feeding assay, the low number of animals precludes definitive conclusions.

Given the absence of exploratory effects, the optical self-stimulation observed most likely stemmed from reward-related processes, although addition assays would enrich interpretation of the ChR2 animals’ preference. If stimulation promotes perseverative behaviors, absent of any appetitive influence, a conditioned place preference (CPP) assay would likely reveal this effect. CPP is a three day assay that requires a three-chamber apparatus, in which one chamber is linked...
to optical stimulation and another to no stimulation. The animal is exposed to each of these chambers, one per day, and on the third day allowed to freely explore the apparatus, with optical stimulation withheld. The animals’ preference for the stimulated chamber on the third day, reflected by increased time within that chamber, is thought to indicate an appetitive effect of stimulation, while avoidance implies an aversive effect. If optical stimulation of BLA to LH prevents the animal from switching strategies, but has no appetitive effect, animals will exhibit no preference for the stimulated chamber, and if aversive, will seek out the nonstimulated chamber. In contrast, in a real-time place preference (RTPP) assay, such animals might still show a preference for optical stimulation, due to an inability to leave the chamber paired to simulation. In RTPP, a single day assay, animals are allowed to freely explore all chambers of the apparatus, in which one chamber triggers optical stimulation. Thus, the CPP assay could help substantiate this projection’s role in reward-related behavior, although it would not differentiate incentive salience (wanting) versus hedonic impact (liking) (Berridge et al., 2009). Assays with analysis for facial ‘liking’ expressions could help distinguish these psychological processes, although studies of CS-potentiated feeding assays and LH (Holland et al., 2002, 2002; Petrovich et al., 2005) indicate a more likely influence upon incentive salience than hedonic impact.

*Analysis of excluded animals indicates possible opposing effect of CEA to LH projection*

Of note, a subset of animals that were excluded from primary analysis due to viral leakage did not exhibit ICSS, despite ample transduction of BLA neurons. This absence of ICSS appeared to occur when virus leaked into the CEA, but not the putamen, raising the possibility that the CEA to LH projection opposes the effect of the BLA to LH projection. In contrast to BLA, CEA contains long range GABAergic projections (Sah et al., 2003), such that the CEA may directly inhibit the neurons that BLA synapses onto, or the CEA may indirectly block ICSS through inhibition of another subset of LH neurons. If this result persists with more animals, ex
*vivo* intracellular recordings in slice may be warranted to untangle the circuit dynamics, particularly if opsins with non-overlapping wavelengths (C1V1 and ChR2) (Yizhar et al., 2011) could be used to activate each projection separately in the same animal while recording from LH neurons.

*Activation of BLA fibers in LH do not nonspecifically trigger feeding*

Animals in familiar environments are known to preferentially interact with novel objects as opposed to familiar objects, which has suggested an appetitive aspect to novelty exploration (Bevins et al., 2002). Thus, given the implication of LH in feeding and reward, I conducted a modified novel object assay. To test for possible effects upon reward processing, feeding, and anxiety-related behaviors, I optically stimulated BLA terminals in the LH during exposure to a novel object and food pellet. ChR2 animals did not exhibit an appreciable difference in novel object exploration or food interaction with and without optical stimulation. However, experimental animals did show a low baseline exploration level, also reflected in the low difference interaction score, especially as compared to eYFP animals. Most likely, this low baseline is an artifact of the low number of animals, perhaps with the eYFP baseline inordinately raised by the performance of a single animal. A true effect of optical stimulation on ChR2 animal’s baseline appears unlikely, given a reduction in center zone exploration was not observed during the OFT, which reflected more animals. Other possibilities include an effect of illumination upon memory, with stimulation on the first day affecting behavior on the second, but counterbalancing of order would most likely dilute this effect. The order of assays could also affect baseline exploration, with optical stimulation in previous assays (OFT and ICSS) producing plasticity within the circuit, which may warrant separate groups of naive experimental animals in future experiments.
Familiar food was employed in this assay instead of a familiar object, the main-stay of novel object assays, in order to gauge consummatory behaviors. Optical stimulation of GABAergic VTA-projecting LH neurons is known to trigger gnawing behavior in a very pronounced fashion (Nieh et al., 2015), and by including both an object and food, I could assay the occurrence of any feeding behavior and whether gnawing motions were food specific. Optical stimulation did not trigger any gnawing or feeding behavior in this assay or any others, which indicates that BLA fibers in LH most likely do not solely project on to GABAergic VTA-projecting LH neurons, given that BLA projections are glutamatergic. (LH-projecting BLA neurons could still potentially project to this population, with synapses onto other neurons eliminating gnawing behavior.)

This absence of feeding behavior also enriches interpretation of previous studies of CS-potentiated feeding. Even though lesions did not affect baseline food consumption, it was unclear whether BLA to LH simply triggers feeding in any circumstance, or performs another function, such as endowing the CS with reinforcing properties, thus causing the animal to pursue the US despite energy-related signals indicating satiation (Baxter and Murray, 2002; Holland et al., 2002; Petrovich et al., 2002). While only a few mice, the lack of any feeding behavior supports the idea that LH-projecting BLA neurons help a CS acquire reinforcing properties, rather than nonspecifically triggering feeding.

**LH anatomy necessitates more specific optogenetic methods**

Although transduction of the entire BLA and implantation of an optic fiber over terminals has permitted selective manipulation of projections in previous studies (Felix-Ortiz and Tye, 2014; Felix-Ortiz et al., 2013; Stuber et al., 2011), the anatomy of the BLA to LH projection most likely requires a more specific method. In addition to LH, the BLA possesses a substantial projection to the VMH (Petrovich et al., 2001), such that an optic fiber over LH may activate
fibers terminating in VMH. As a control for such an effect, many studies conduct optical stimulation prior to perfusion and stain for immediate early genes, such as c-fos (Lammel et al., 2012; Tye et al., 2011). However, the LH sends a small projection to VMH (Ter Horst and Luiten, 1987), such that c-fos expression in VMH could represent activation directly via ChR2, or secondary activation by LH neurons responding to illumination. A glutamate receptor antagonist could be infused into the LH prior to ICSS, and any resulting absence of optical self-stimulation would strongly substantiate the role of LH-projecting BLA neurons in motivated behavioral responding. However, the close proximity of the VMH, immediately adjacent to the LH may obfuscate conclusions, and a dye infused along with the antagonist may not spread in the same manner as the drug or persist in the tissue until perfusion, preventing confirmation of glutamate blockade in the LH alone.

The ChR2 animals exhibited a very short window in which optical stimulation supported ICSS but did not provoke seizures, highlighting the need for controls for possible antidromic activation. It is unclear whether LH-projecting BLA neurons possess collaterals, or where such collaterals terminate, but infusion of lidocaine into the BLA during ICSS would block such an effect, while still permitting optical activation of terminals in LH. However, given the technical difficulties concerning fibers of passage, discussed above, I proceeded to targeting the projection with another method rather than attempting pharmacological inactivation of BLA cell bodies.

**Preliminary results with cre-DIO method**

Given the aforementioned confounds, I tried to selectively transduce only LH-projecting BLA neurons using a cre-DIO method. Previous retrograde viruses, such as HSV-cre, produced minimal penetrance in our lab, even after lengthy incubation times (6 months or more), while quicker methods, involving a pseudorabies virus, often produced toxicity before all control
assays could be completed. As a result, I opted to infuse a CAV2-Cre virus into the LH, which, as shown by the confocal imaging of an optrode animal, resulted in expression of ChR2 in a subset of BLA neurons. However, the absence of a fluorophore in this virus still creates difficulties due to the proximity of VMH, so I simultaneously infused a virus bearing a fluorophore alone, under the CamKII promotor. A second virus may still spread differently than the CAV2-cre virus, although it would most likely produce a closer approximation than dye and persist in the tissue long enough for histological examination. In future experiments, a fluorophore under an even less specific neuronal promotor may also be preferable.

With this method and an optic fiber implanted over the BLA, I did not observe optical self-stimulation in the ICSS assay, however, several limitations of this study mean that LH-projecting BLA neurons may still promote motivated behavioral responding. First and foremost, incorrect placement of the viruses or the optic fiber could explain the absence of an effect. The previous method may have also targeted a different subset of LH-projecting BLA neurons than the cre-DIO method, as a single optic fiber cannot illuminate the entire LH, given its lengthiness. The virus may have spread further than the light cone in previous experiments, and BLA neurons projecting to different parts of LH may have opposing effects, resulting in an absence of a result with ICSS. Repetition of the experiment with infusion of a smaller volume of virus may be warranted, and dissection of differing effects depending on the projection target along LH could be fruitful. If further experiments with the cre-DIO method show optical self-stimulation, it is likely that LH-projecting BLA neurons support motivated behavioral responding.

*Future directions for investigating the encoding properties of LH-projecting BLA neurons*

In order to characterize the encoding properties of this subset of neurons, I selectively transduced LH-projecting BLA neurons with the cre-DIO method and implanted an optrode in the BLA, so that LH-projecting BLA neurons could be identified via their response to
illumination. After recovery, the animal was subjected to a partial reinforcement operant conditioning task, which was used due to previous demonstration of deficits with this task following NMDA blockade of the BLA (Tye et al., 2008), making the BLA’s involvement likely. I also employed this task rather than a Pavlovian task because the animal’s initiation of each trial helps control for attentional effects.

During surgery, I recorded from photoresponsive units, but these units did not persist in behavioral recording sessions. However, an animal successfully learned the task while connected to a headstage and patch cable, as evidenced by the learning curve, which is taken from the second day of recording. The animal’s performance begins above 50% (that expected by chance) most likely due to learning during the first day’s session. The identification of photoresponsive units during surgery also improves upon previous methods with HSV-Cre in our lab, which had produced such sparse labelling in the BLA that few photoresponsive units appeared in recordings. Thus, while I did not record phototagged units during the actual task, I did demonstrate the feasibility of this experiment, which merits further investigation given my preliminary results with ICSS and this projection.

Possible firing patterns among LH-projecting BLA neurons during an appetitive task

LH-projecting BLA neurons may exhibit several forms of encoding in the context of appetitive learning. Previous electrophysiological recordings during appetitive conditioning and operant tasks have demonstrated pronounced diversity in the firing activity of BLA neurons, with responses to rewards (unconditioned stimuli (US)), reward-predictive cues (CS) (Muramoto et al., 1993; Paton et al., 2006; Sanghera et al., 1979; Tye and Janak, 2007; Tye et al., 2008; Uwano et al., 1995), expectation of reward (Schoenbaum et al., 1998), and omission of expected reward (Roesch et al., 2010; Tye et al., 2010). The effect of contralateral BLA and LH lesions on CS-potentiating feeding indicates that LH-projecting BLA neurons may evoke the value of the CS
(Petrovich and Gallagher, 2003). Other electrophysiological studies in the primate and rat have suggested that BLA neurons encode current stimulus-value associations (Baxter and Murray, 2002; Belova et al., 2008; Paton et al., 2006; Schoenbaum et al., 2007) rendering it likely that LH-projecting BLA neurons will respond to reward-predictive cues in an appetitive conditioning task.

If LH-projecting BLA neurons encode the value of reward-predictive cues, units may display a phasic response to the US during initial recordings, followed by response to the CS and US as the animal learns, with depolarization due to the US allowing for NMDA-dependent plasticity. After acquisition, such neurons may decrease firing over the course of the session, as the value of the outcome diminishes due to satiation. LH-projecting neurons may also respond to the presence or absence of sucrose, regardless of expectation, as found in previous studies of BLA neurons (Tye et al., 2010), or they may represent motor activity related to conditioned approach. If such activity is observed, recordings in animals with unpaired sucrose and tones will potentially help differentiate activity related to movement execution and reward expectation.

After acquisition, recordings could also occur while altering the animal’s learned associations, such as delivering greater sucrose than expected or omitting expected sucrose. Such manipulations would allow for further analysis of value coding and signed and unsigned reward prediction error (RPE). If LH-projecting BLA neurons exhibit value coding, then the response will diminish across blocks in which the tone no longer predicts sucrose delivery, and possibly increase with greater sucrose. If these neurons encode signed reward prediction error (RPE), units will exhibit inhibition to sucrose omission and an increase in firing to greater sucrose delivery (Schultz, 2006). During blocks, this response will diminish as the outcome becomes expected, with perhaps greater firing transitioning to the cue in blocks involving greater sucrose delivery. Rather than signed prediction error, previous recordings have reported a subset of BLA
neurons that appear to encode unsigned prediction error, in which an unexpected outcome increases firing, regardless of valence (Roesch et al., 2010). If LH-projecting BLA neurons encode unsigned prediction error, the omission of sucrose or delivery of greater sucrose will both increase firing, and this firing will diminish across the block. Furthermore, LH-projecting BLA neurons may all show similar firing properties, as reported with VTA-projecting LH neurons (Nieh et al., 2015), or they may show heterogeneity across neurons or a combination of encoding properties within a single unit.
Conclusion

Although both the BLA and LH have been implicated in reward processing, pre-existing techniques have rendered it difficult to dissect the function of this projection in appetitive operant conditioning. Through optogenetic manipulations, I have found preliminary evidence that LH-projecting BLA neurons support motivated behavioral responding, and I have demonstrated the use of in vivo extracellular recordings with identification of these neurons via their photoresponse. Through a more specific method of manipulating the BLA to LH projection, future experiments could substantiate these preliminary findings, as well as delve into the encoding properties of this subset of BLA neurons.
References


