UNIVERSITY OF CALIFORNIA SAN DIEGO

Selective Neuromodulation of Medial Prefrontal Cortex Inputs to the Lateral Habenula by Norepinephrine

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The thesis of E-Jane Tsai is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

For my family

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For my friends who have become family

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For the one who continuously hyped me up to be a neuroscientist at the forefront of my field

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Roberto Malinow, Chair

Due to the firing of lateral habenula neurons (LHb) biasing a negative perspective of the world, an increase in LHb activity has been implicated in causing depression. Inputs to the LHb from higher-level cognition areas are likely providing the necessary signals for the LHb to respond to aversive stimuli and subsequently increase in activity. Projections to the LHb from the medial prefrontal cortex (mPFC) that processes negative valence events, have been identified, but the functions and characteristics of mPFC inputs to LHb still remain unknown. Here, we have identified and characterized mPFC inputs to LHb to be excitatory,

glutamatergic, and under selective neuromodulation by norepinephrine (NE). Inputs from two other brain regions that may be instructing the LHb to process aversive stimuli, the ventral tegmental area (VTA) and the lateral hypothalamic area (LHA), were not affected by the addition of NE. We therefore propose that the lack of effect by NE on both VTA and LHA inputs may be due to the activation of adrenergic receptors at mPFC terminals, but not at VTA or LHA terminals, facilitating increased glutamate release.

INTRODUCTION

1.1 Lateral Habenula and Depression

Major Depressive Disorder (MDD) is psychological disorder that is a leading cause of both disability and mortality, affecting over a hundred million people worldwide (Reddy, 2010). Anhedonia, a common symptom of depression characterized by a loss of motivation or pleasure, points to MDD affecting reward perception (Admon and Pizzagalli, 2015). Dysfunctions in the reward processing mechanism of depressed patients reduces their response to rewarding stimuli, likely inducing depression (Admon and Pizzagalli, 2015).

One brain region involved in reward processing and has been implicated in depression is the lateral habenula (LHb) (Proulx et al., 2014). The LHb is known as a negative-reward processor that responds to aversive stimuli, such as reward omission or presentation of punishment (Proulx et al., 2014). In both animal models of depression and in humans, depression is correlated with an increase in LHb activity (Yang et al,, 2018). If the LHb encodes for disappointment, then an increase in the firing of LHb neurons is thought to contribute to depression by continuously biasing a negative view of the world (Proulx et al., 2014). Identifying and characterizing (e.g. neuromodulation of) the inputs to the LHb that instruct the LHb to respond to aversive stimuli can thus lead to a better understanding of what contributes to LHb hyperactivity.

1.2 Lateral Habenula as a Negative-Reward Processor

Reward perception serves a behavioral function of motivating organisms to assess and interact with its environment (Schultz et al., 1997). How motivated an animal will be in

partaking in a task is predicted by the difference between the perceived expectation of a reward and the actual value of a reward received, referred to as reward prediction error (RPE) (Schultz, 2016). Positive RPE indicates a reward received was greater in value than what was expected and would motivate future action in animals while negative RPE indicates less reward was received than expected and would discourage future action (Schultz, 2016). As a decrease in motivation is characteristic of anhedonia, those suffering from depression may suffer from an increase in negative RPE perception that biases feelings of disappointment.

The LHb is implicated in depression because it signals negative RPE (Matsumoto and Hikosaka, 2007, Matsumoto et al., 2009, Browne et al., 2018). From monkeys trained to recognize a non-reward and reward-indicating tone, LHb neuron activity was observed to increase when reward was omitted (negative RPE) and decrease when reward was expected (positive RPE) (Matsumoto and Hikosaka, 2007). Firing of LHb neurons also inhibited midbrain dopamine neurons that responded actively to the reward-predicting tone (positive RPE) (Schultz et al., 1997, Matsumoto et al., 2007). When dopamine neurons in the ventral tegmental area (VTA) were inhibited in rats, a depressive-like behavior emerged where the rats decreased their consumption of a sucrose reward given upon the completion of a trained task (van Zessen et al., 2012). Due to dopamine neurons being responsible for signaling the expectation of positive outcomes, inhibition of dopamine neurons by the LHb (see below) prepares animals to expect the worst, and the ensuing disappointment may contribute to the development of depression van Zessen et al., 2012).

1.3 Lateral Habenula Circuitry

Inhibition of VTA dopamine neurons by the LHb can occur either through a direct or indirect pathway (Christoph et al., 1986, Omelchenko et al., 2009, Hong et al., 2011). The direct pathway involves LHb neurons sending excitatory glutamatergic projections that synapse onto inhibitory GABAergic neurons in the VTA (Ji and Shepard, 2007, Omelchenko et al., 2009). When LHb activity increases in response to negative stimuli, GABAergic VTA neurons inhibit dopamine neuron activity (Ji and Shepard, 2007, Omelchenko et al., 2009, van Zessen et al., 2012).

The indirect pathway involves LHb neurons sending glutamatergic projections to an intermediary structure between the LHb and VTA, the rostromedial tegmental nucleus (RMTg) (Hong et al., 2011, Balcita-Pedicino et al., 2011). The RMTg sends inhibitory GABAergic projections to the VTA and when LHb activity increase in response to negative stimuli, excited RMTg neurons inhibit VTA dopamine neurons (Hong et al., 2011, Balcita-Pedicino et al., 2011). Animals receiving an aversive stimulus, foot shocks, show increased activity in RMTg projecting neurons (Stamatakis and Stuber, 2012). Stimulating RMTg projecting LHb neurons also leads to avoidance behavior, consistent with the LHb as a driver of aversive behavior (Proulx et al., 2018, Shabel et al., 2012, Stamatakis and Stuber, 2012).

As increased firing of the LHb to aversive stimuli inhibits monoaminergic systems and induces depressive-like behavior, multiple studies suggest that LHb hyperactivity may be a cause of depression (Hu and Fitzgerald, 2018, Proulx et al., 2014). Compared to wildtype animals, learned helpless animals, an animal model of depression, show hyperactivity in the excitatory synapses of neurons in the LHb (Li et al., 2011). The cellular mechanism

responsible for an overactive LHb triggering depression appears to involve dysfunctions in the neurotransmission of the excitatory neurotransmitter glutamate in the LHb (Hu and Fitzgerald, 2018). Thus, asking what inputs to the LHb affect glutamate release can reveal how LHb hyperactivity occurs (Shabel et al., 2012).

Identifying excitatory inputs to the LHb can help us understand contributors to LHb hyperactivity. So far, our lab has found a synaptic potentiation onto VTA projecting LHb neurons in a rat model of depression when compared to wild type rats (Li et al., 2011). We have also found that excitatory inputs from the basal ganglia, a brain region involved in reward function, to the LHb are responsible for signaling LHb response to aversive stimuli (Shabel et al., 2012, Schultz, 2016). Furthermore, stimulation of these excitatory basal ganglia inputs to the LHb causes aversion in rats where the rat will avoid the compartment where stimuli were received (Shabel et al., 2012). Lastly, serotonin, a neuromodulator related to depression, suppresses the excitatory basal ganglia inputs to the LHb (Shabel et al., 2012). Taking inspiration from the Shabel and colleagues (2012) study, we are now investigating inputs to the LHb from the medial prefrontal cortex (mPFC), a brain region thought to be involved with negative emotion (Etkin et al., 2011, Riga et al., 2014).

1.4 Medial Prefrontal Cortex Inputs to the Lateral Habenula

Although studies have provided evidence for the existence of projections from the mPFC to LHb, there are still few studies on the functions of these projections (Greatrex and Phillipson, 1982, Kim and Lee, 2012). The mPFC provides higher-level cognitive input including processing and expression of negative emotion (Etkin et al., 2011, Riga et al.,

2014). Such findings suggest that the mPFC may produce cognitive and emotional deficits present in Major Depression Disorder (Etkin et al., 2011, Riga et al., 2014). Inputs from the mPFC to LHb that affect dopaminergic systems would allow mPFC regulation of emotional behavior (Kim and Lee, 2012). One study on a prefrontal-habenular pathway has that found stimulating mPFC axons in the LHb of rats reduces mobility during forced swim test (FST), a behavioral assessment correlating depressive-like behavior with animal immobility when the animal is placed in a cylindrical container of water (Warden et al., 2012). This reduction in mobility was similar to a recent finding in our lab where an increased LHb drive onto the RMTg in rodents reduces the motivation to exert action during FST (Proulx et al., 2018). It could have been that mPFC inputs were acting on RMTg projecting LHb neurons to decrease mobility in FST and if so, would suggest mPFC inputs to the LHb are involved in mediating depressive-like behavior.

To identify and characterize mPFC inputs to LHb, a combination of optogenetics with two-photon calcium imaging was used to stimulate mPFC axons in the LHb of rats and image LHb neuron activity. In addition to mPFC inputs being excitatory and glutamatergic, we found that addition of norepinephrine (NE) significantly potentiated the synaptic inputs from mPFC to LHb. Consistent with the view that mPFC regulates emotional behavior via inputs to the LHb affecting dopaminergic systems, we also found a potentiation onto RMTg and VTA projecting LHb neurons when mPFC terminals were stimulated in the presence of NE. Next, we examined if inputs from other brain regions exerting influence over LHb response to aversive stimuli, the VTA and lateral hypothalamic area (LHA), were affected by NE (Lecca et al., 2017). Neither VTA nor LHA terminal stimulation in the presence of NE significantly

increased synaptic transmission onto LHb, suggesting that the potentiating effect of NE was selective to mPFC inputs to LHb. We propose that NE exerts its potentiating effects by facilitating glutamate transmission in the LHb, and that its selective neuromodulator effects on mPFC inputs to LHb are due to specific NE binding sites at mPFC terminals absent at both VTA and LHA terminals.

MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats were kept 1-3/cage under a 12/12 hour light/dark cycle (9am/9pm) and given 24/7 access to food and water. At 3-4 weeks old, they underwent live-surgery for injection of AAVs and were sacrificed at 8-12 weeks for slice preparation for use in two-photon calcium imaging experiments. All procedures involving animals were approved by the Institute Animal Care and Use Committees of the University of California, San Diego.

2.2 Stereotaxic Injection of AAVs

Standard stereotaxic injection procedures for rodents were followed. To study mPFC synaptic inputs to the LHb, we injected a virus expressing red fluorescent channelrhodopsin protein (AAV-oChIEF-tdTomato) into the mPFC and a virus expressing green fluorescent calcium indicator (AAV-GCaMP6F or AAV-Flex-GCaMP6s) into the LHb. A cre-dependent viral vector expressing calcium indicator (AAV-Flex-GCaMP6s) was used to selectively label VTA or RMTg projecting LHb neurons with the injection of a retrogradely traveling viral vector expressing cre recombinase (AAV-hSyn-Retro-Cre) in either the VTA or RMTg. To study VTA synaptic inputs to the LHb and LHA synaptic inputs to the LHb, a virus expressing channelrhodopsin (AAV-oChIEF-tdTomato) was injected into either the VTA or LHA with injection of a virus expressing calcium indicator (AAV-GCaMP6F) into the LHb.

A stereotaxic apparatus and ‘Angle Two’ computer system was used for bilateral injection of AAVs into rats, aged 3-4 weeks and anesthetized with isoflurane. Subcutaneous

injection of a local anesthetic, bupivacaine, was given prior to exposing the skull. The skull was drilled at the following injection site coordinates: LHb [anterior-posterior (AP): -3.4 mm, medial-lateral (ML): ±0.65 mm, dorsal-ventral (DV): -4.45 mm], mPFC [AP: 2.6-2.7 mm, ML: ±0.36 mm, DV: -1.5 - -3.5 mm], VTA [AP: 5.3 mm, ML: ±0.8 mm, DV: -7.8 mm], and LHA [AP: -1.8 mm ML: ±1.9 mm, DV: -9.0 mm]. A glass pipette containing 1µL of virus was connected to a picrospritzer that applied pressure to inject 0.5µL of virus into each hemisphere. To ensure diffusion of virus after injection, we allowed 5 minutes to elapse before the pipette was removed. A subcutaneous injection of 5mg/kg carprofen was given after surgery and animals were allowed to recover in a heated recovery cage. A heated pad was placed under animals during surgery as well.

2.3 Acute Brain Slice Preparation

Full expression of viral product was assumed to be reached 4-8 weeks following surgery, at which time rats were sacrificed for acute brain slice preparation. Brains were removed from decapitated rats anesthetized with isoflurane. Using a vibratome, 400µm coronal/sagittal slices with the LHb visible were made in an ice-chilled cutting buffer (25.0 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM KCl, 0.5 mM $CaCl_2$, 10.0 mM $MgSO_4$, 25.0 mM glucose, pH 7.2-7.4; gassed with 95%O_2/5%CO_2). Slices were placed in a chamber containing 35°C cutting buffer for 12 minutes before being transferred into artificial cerebrospinal fluid (ACSF) (119 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 12.5 mM Glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$; 22°–25°C; gassed with 95%O_2/5%CO_2) and allowed to recover at

room temperature in ACSF for 40-60 minutes before recording from them. Figure 1 shows the schematic of methods for example experiments performed to study mPFC inputs to LHb.

2.4 Two-photon Calcium Imaging

A Bruker two-photon laser resonant scanning microscopy with Bruker's Prairie View software was used to image LHb activity with and without optical stimulation to mPFC/VTA/LHA synapses expressing channelrhodopsin. Slices were placed under a 20x water-immersion Olympus XLUMPlanF 0.95 NA objective in a recording chamber that received continuous perfusion of ACSF. We recorded LHb activity in response to optical stimulation of synapses expressing channelrhodopsin for the following three experimental conditions: a baseline response before norepinephrine (NE) addition, response to the addition of 5µM NE, and response after NE was washed out of the perfusion.

For all experimental conditions, recordings with and without optical stimulation were taken for each experimental condition. First, the brain slice was imaged for 100 frames with no optical stimulation to observe for spontaneous activity in LHb neurons. Next, a 20 cycle imaging session was done with 25 frames/cycle in which a single light pulse (10 ms, 473 nm) was delivered every 13^{th} frame in each cycle. A brain slice was imaged twice under baseline response conditions with the second set of recordings taken 20 minutes after the first, to allow us to later normalize NE and wash data points by the average of two baselines. To test effects of NE on mPFC inputs to LHb, 5µM NE was added to the ACSF perfusion and the perfusion was allowed to circulate for 5 minutes before the same recordings with and without optical stimulation were taken. Lastly, NE was washed out by draining the circulating

ACSF solution containing NE and circulating new ACSF several times through the perfusion before the last set of recordings were taken.

2.5 Norepinephrine Release

Standard stereotaxic surgery procedures for rodents as mentioned in chapter 2.2 was followed. We bilaterally injected a virus expressing NE sensor (AAV-hSyn-GRAB$_{NE1}$) into the LHb and a virus expressing channelrhodopsin (AAV-oChIEF-tdTomato) into the locus coeruleus (LC) (AP: 10 mm, ML: 1.5mm, DV: 6.1 mm). Rats were sacrificed and brain slices were prepared according to the methods outlined in chapter 2.3. Slices were placed in a recording chamber receiving a constant perfusion of ACSF and imaged using a two-photon laser.

We first observed for spontaneous NE release in the LHb by imaging 500 frames without any optical stimulation. To test whether LC synapses in the LHb can be evoked to release NE, a 10 cycle imaging session was done with 25 frames/cycle in which a single light pulse (10 ms, 473 nm) was delivered at every 13th frame. The sensitivity of the sensor was tested by adding different concentrations of NE (1µm, 3µm, 5µm) to the ACSF perfusion and waiting 5 minutes for the drug to circulate completely before taking both with and without optical stimulation recordings. The increase in fluorescence intensity was plotted against the concentrations of NE added and the concentration of NE released by LC stimulation was roughly estimated by comparing fluorescence intensity of NE release to fluorescence intensity of NE concentration added.

2.6 Data Analysis

All recorded images in Bruker's Prairie View were downloaded to and saved in ImageJ as tiff files. Custom-written MATLAB codes were used to motion correct the images and analyze neuron responses using a whole window regions of interest (ROIs) approach that plotted the average LHb activity in each frame over 20 cycles. The single light pulse was delivered at the 13^{th} frame of each cycle and the response of LHb to stimulation of mPFC synapses was plotted as an increase in amplitude peak. For each experimental condition, the average of the difference from the amplitude peak to a point before and after the 13^{th} frame was calculated and considered to be LHb response to optical stimulation of mPFC inputs.

All responses from the same slice recording was normalized by the average baseline of that slice. As we were interested in whether excitatory inputs were potentiated, student's 1-tailed t- test was used to compare 'baseline' and 'NE' values with $p < 0.05$ indicating a significant potentiation by NE. The average of all normalized responses from all slice recordings for each experimental condition was graphed as mean ± SE.

RESULTS

3.1 mPFC inputs to LHb are potentiated by NE

To study mPFC inputs to LHb, a virus expressing a green fluorescent calcium indicator protein (AAV-GCaMP6F) was injected into the LHb for subsequent two-photon calcium imaging and a virus expressing a red fluorescent channelrhodopsin protein (AAV-oChIEF-tdTomato) was injected into the mPFC for optical stimulation of mPFC inputs to LHb. These injections did not specify the type of LHb neurons we were recording from and will be referred to as mPFC inputs to LHb (unspecified projections). Accurate injection sites were confirmed using a dissecting microscope to observe for green fluorescence in the LHb and red fluorescence in the mPFC (Fig 1B). After 4-8 weeks, rats were sacrificed and brain slices were prepared for two-photon laser imaging, in which we confirmed green fluorescent neuron cell bodies and red-fluorescent fibers in the LHb (Fig 2A left, middle). The overlapping fluorescence indicated that LHb neurons receive dense innervation from mPFC axonal terminals (Fig 2A right).

Using optogenetics and two-photon calcium imaging, we stimulated mPFC axonal terminals in the LHb while imaging LHb neuron activity (Figure 1). We found mPFC inputs to LHb (unspecified projections) were excitatory and glutamatergic, as addition of an AMPA antagonist, NBQX, eliminated excitatory response of LHb to stimulation of mPFC terminals (Figure 6A). The relatively small response amplitude when mPFC terminals were stimulated (Table 1), however, indicated that mPFC inputs to LHb were weak and led us to ask if mPFC inputs to LHb could be potentiated, possibly by a neuromodulator. We found that addition of norepinephrine (NE) significantly potentiated mPFC inputs (Figure 2C top) as indicated by

student's 1-tailed t-test (n=17, t=1.76, *p=0.011) (Fig 2C top). The effects of NE, however, were eliminated when NE was washed out, indicating only a transient synaptic potentiation onto LHb neurons (Fig 2B middle, bottom).

After finding that NE potentiated excitatory mPFC synaptic inputs to LHb (unspecified projections), we asked if this synaptic potentiation onto LHb neurons was selective to the type of LHb neuron. Of interest were the LHb neurons that project to the ventral tegmental area (VTA) and rostromedial tegmental nucleus (RMTg), as they cause the inhibition of dopaminergic neurons (van Zessen et al., 2012, Stamatakis and Stuber, 2012). To select for these specific LHb neurons, we injected a cre-depedent virus expressing a calcium indicator (AAV-Flex-GCaMP6s) into the LHb and a retrogradedly traveling virus expressing cre-recombinase (AAV-hSyn-Retro-Cre) in either the VTA or RMTg. We found that NE also increased mPFC inputs to both VTA projecting (n=10, t=1.83, *p=0.040) and RMTg projecting (n=10, t=1.83, *p=0.045) LHb neurons (Fig 2C bottom right, left).

3.2 VTA and LHA inputs to LHb are not potentiated by NE

Our findings that NE potentiated synaptic inputs from the mPFC to the LHb led us to ask if NE has an excitatory effect on synaptic transmission when the terminals of other brain regions with excitatory inputs to the LHb are stimulated. Of interest were the VTA and lateral hypothalamic area (LHA), both of which have been identified to have excitatory inputs to the LHb (Gruber et al., 2007, Poller et al., 2013). The same two-photon calcium imaging and optogenetic experiments were performed to study VTA and LHA inputs to the LHb. Addition of NE did not cause an observable increase in LHb neuron response to stimulation of either

VTA or LHA terminals (Fig 3B & Fig 4B) and student's 1-tailed t test indicated a lack of significance as well (VTA: n=15, t=1.76, p=0.24, LHA: n=5, t=2.13, p=0.25) (Fig 3C & 4C). The lack of an excitatory effect by NE on both VTA and LHA inputs to the LHb led us to conclude that there was a selective neuromodulation of mPFC inputs to LHb.

3.3 NE release onto LHb from locus coeruleus terminals

After concluding that NE at mPFC axonal terminals increases synaptic transmission in LHb, we were interested to know if this phenomenon can occur *in-vivo*. We began with investigating sources of NE release in the LHb, particularly looking for noradrenergic innervation from the locus coeruleus (LC), which contains neurons that are the primary site of NE synthesis and release (Atzori et al., 2016). A virus expressing channelrhodopsin (AAV-oChIEF-tdTomato) was injected into the LC (Fig 5A) and a virus expressing NE sensor (AAV-hSyn-$GRAB_{NE1}$) into the LHb. Literature indicates that the $GRAB_{NE1}$ sensor is expressed in all parts of the neuron, including the cell bodies, dendritic-shafts, spines, and axons (Feng, 2019). This expression pattern appeared to be the case in LHb neurons as two-photon laser imaging revealed NE sensor expression throughout the LHb (Fig 5B left & 5C left).

In a 500 frame recording of the LHb without optical stimulation, we were able to identify a neuron with fluctuating levels of fluorescence intensity, indicating that NE release was occurring spontaneously in the LHb (Fig 5B left). We then sought to find if we could induce NE release in the LHb by stimulating LC axon terminals. A single pulse of light (10 ms, 473 nm) was delivered every 13^{th} imaging frame and in the neuron that was observed to spontaneously detect NE, increases in fluorescence intensity was observed in alignment with

the time of stimulation at every 13th frame (Fig 5B right). This indicated that stimulation of LC terminals was causing subsequent NE release from LC terminals in the LHb.

Next, we added varying concentrations of NE to the perfusing ACSF to confirm that the sensor was responding to a presence of NE and to test the sensitivity of the NE sensor (Fig 5C). Fluorescence intensity increased each time total NE concentration increased and when all NE was washed out, fluorescence levels returned to baseline before any NE addition occurred (Fig 5C right). From these increases in fluorescence after the addition of external NE, we confirmed that the fluorescence increases observed in Figure 5B were indeed due to a release of NE in the LHb being detected by the NE sensor. By plotting the fluorescence intensity of each NE concentration versus the concentration of NE, we were able to roughly estimate the concentration of induced NE release from LC terminals to be less than 0.5 μM (Fig 5D). The exact concentration of NE release could not be calculated because once NE was released, NE diffused into the extracellular space and as a result, the NE concentration detected at the sensor was diluted. However, from our tests of sensor sensitivity, we can reasonably conclude that less than 1 μM NE release occurs from LC terminals in the LHb.

DISCUSSION

As the LHb is excited by negative valence events and the mPFC is involved in various higher cognitive functions including encoding emotional valence and regulating emotion-related behaviors, the mPFC could be sending top-down modulatory signals to the LHb (Proulx et al., 2014, Etkin et al., 2011, Riga et al., 2014). We found that mPFC inputs to LHb were excitatory, glutamatergic, and could be potentiated by addition of NE (Figure 2 & 6A). Having characterized mPFC inputs to LHb to be largely glutamatergic, we hypothesize that NE could be facilitating the release of the excitatory neurotransmitter glutamate at mPFC axonal terminals. A previous study stated LHb activity is determined by the ratio of inhibitory GABA to excitatory glutamate release in the LHb (Shabel et al., 2014). Therefore, LHb output would be expected to increase from a facilitation of glutamate transmission in the LHb by NE acting at mPFC terminals.

We propose the site of NE action is presynaptic, increasing a release of glutamate at mPFC terminals. A study of NE in the rat olfactory cortex revealed similar results as ours where bath application of low concentrations (0.1 – 5 uM) of NE increased excitatory transmission at the lateral olfactory tract (Collins et al., 1984). The study also concluded that the excitatory effects of NE on synaptic transmission was due to low concentrations of NE facilitating a release of excitatory transmitters, including glutamate (Collins et al., 1984). Our results that 5 uM NE at mPFC terminals is sufficient to facilitate LHb response (Figure 2) is consistent with the excitatory effects of low concentrations of NE observed in the rat olfactory cortex (Collins et al., 1984). Previously, we have indicated the involvement of AMPA receptors in mediating mPFC inputs to LHb (Figure 6A), and increased activation of

AMPA receptors in the LHb is correlated with an overactive LHb (Hu and Fitzgerald, 2018). Assuming NE in our experiment was also facilitating glutamate release as per the results of Collins et al., it is likely that the increased glutamate was activating AMPA receptors in the LHb to increase LHb activity. Despite studies pointing toward a presynaptic effect of NE, we cannot exclude the possibility that NE might have a postsynaptic site of action as well. In this case, we would expect NE to increase postsynaptic glutamate receptor opening, most likely AMPA receptors due to our results of NBQX eliminating excitatory responses (Figure 6A).

Interestingly though, NE did not potentiate ventral tegmental area (VTA) inputs or lateral hypothalamic area (LHA) inputs to LHb (Figure 3 & 4), two brain regions that provide aversive information to the LHb (Lecca et al., 2017, Montardy et al., 2019). As VTA inputs to LHb were also found to be glutamatergic (Figure 6B) and LHA inputs to LHb were shown to be glutamatergic by Lecca et al., we would have expected the same bath application of low concentrations of NE to facilitate glutamate release and cause subsequent potentiation onto LHb neurons. Under consideration for the lack of significant effect observed is whether mPFC terminals have specific NE binding sites facilitating glutamate release that are absent at both VTA and LHA terminals. Amongst the adrenergic receptors activated by NE, we suspect activation of α1 adrenoreceptors (α1AR) by NE contributes to the selective neuromodulation of mPFC inputs to LHb (see below).

One experiment found persistent firing in the prefrontal cortex (PFC) was due to α1AR on PFC terminals facilitating glutamate release (Zhang et al., 2013). A separate experiment showed high co-localization of α1AR with vGluT1 at axonal terminals in the PFC (Mitrano et al., 2012). Although α1AR were also found at terminals in the VTA, they were

predominantly co-localized with GABA terminals rather than glutamatergic terminals (Mitrano et al., 2012). It could be when VTA→LHb were stimulated, the activation of α1AR at VTA terminals causing GABA release was depressing the excitatory effects of NE facilitating glutamate release. This is consistent with our results that show an increase in LHb response after NE addition, but not a significant potentiation (Figure 3C). Similarly, the lack of significant effect on LHA inputs to LHb may also be attributed to less co-localization of α1AR with glutamatergic terminals. It is important to note the lack of significance for LHA inputs to LHb may be partly due to the relatively small sample size we were able to collect as compared to sample size collected for both mPFC and VTA inputs to LHb.

In our future experiments, we can test the effects of α1AR antagonists on stimulating mPFC, VTA, and LHA inputs to LHb in the presence of NE. Elimination of an excitatory effect when stimulating mPFC inputs to LHb would indicate involvement of α1AR in mediating the excitatory effects of NE. An increase in synaptic transmission when VTA and LHA terminals are stimulated would further confirm α1AR to be responsible for the selective neuromodulation of mPFC inputs to LHb by NE. Co-localizations of VGlut, VGAT, and α1AR in the LHb can be confirmed by immunostaining for them with corresponding antibodies.

In regard to the selective neuromodulation of mPFC inputs to LHb, the excitatory effect of NE on mPFC inputs to LHb included a significantly increased synaptic potentiation onto VTA and RMTg projecting LHb neurons (Figure 2). As LHb projections to both the VTA and RMTg inhibit midbrain dopamine neurons and induce depression (Omelchenko et al., 2009, Hong et al., 2011), potentiation of mPFC inputs to these projector neurons supports a top-down modulation of LHb response to negative valence stimuli by the mPFC (Proulx et al.,

2014, Etkin et al., 2011, Riga et al., 2014). Although we were unable to conduct behavioral experiments on effects of stimulating mPFC inputs to LHb, a study by Warden et al, found stimulation of mPFC terminals in the LHb to increase depressive-like, immobile behavior in rats during forced swim test (FST) (Warden et al., 2012). In separate experiments from our lab, immobility during FST was shown to increase by potentiation of LHb neurons projecting to the VTA and RMTg (Li et al., 2011, Proulx et al., 2018). Our results that VTA and RMTg projecting LHb neurons are affected by mPFC inputs suggest these projector neurons play a role during mPFC→LHb stimulation to cause FST immobility.

In finding that exogenous addition of NE potentiated mPFC inputs to LHb, we must address whether this phenomenon may occur *in-vivo*. So far, we have identified noradrenergic projections from the LC that would provide an *in-vivo* source of NE in the LHb (Figure 5). Moreover, we have observed spontaneous NE release in the LHb and have also been able to evoke NE release by stimulating LC terminals (Figure 5). These are likely indicators that *in-vivo* potentiation of mPFC inputs to LHb could occur by release of NE from LC terminals in the LHb. The next steps in our experiments are to address how the excitatory, glutamatergic mPFC inputs to LHb function *in-vivo* and translate to behavior. Eventually, we hope to determine whether a top-down modulation of LHb by mPFC is responsible for LHb response to negative events.

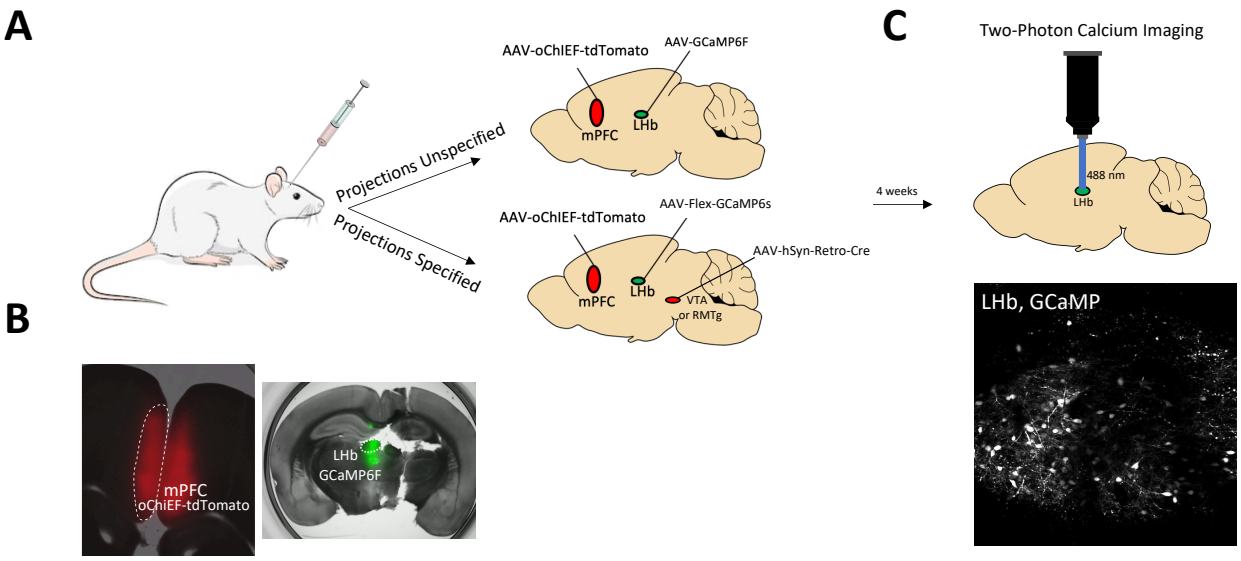

Figure 1. Schematic of Methods. Synaptic inputs to the LHb were studied using optogenetics with two-photon calcium imaging. Shown here is a series of experiments for studying mPFC synaptic inputs to the LHb. (**A**) Rodents underwent stereotaxic injections with a virus expressing channelrhodopsin (AAV-oChIEF-tdTomato) in the mPFC and a virus expressing a calcium indicator (AAV-GCaMP6F) in the LHb. To selectively label VTA and RMTg projecting LHb neurons, a cre-dependent viral vector expressing a calcium indicator (AAV-Flex-GCaMP6s) was injected into the LHb with a retrogradely traveling virus expressing cre-recombinase (AAV-hSyn-Retro-Cre) into either the VTA or RMTg. (**B**) Accurate injection sites were confirmed by dissection microscope images showing oChIEF-tdTomato expression in the mPFC and GCaMP6F expression in the LHb. (**C**) Around 4 weeks later, brain slices containing the LHb were prepared for two-photon calcium imaging.

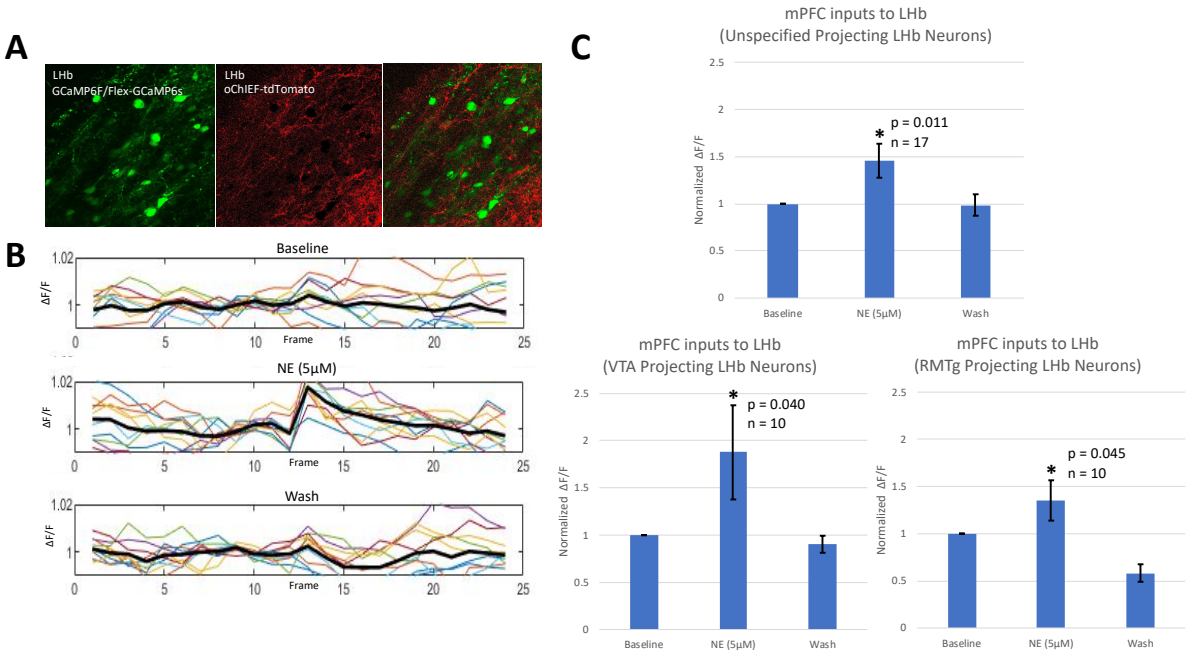

Figure 2. NE potentiates mPFC inputs to LHb. A two-photon calcium imaging experiment was performed to image activity of LHb neurons, expressing a calcium indicator (AAV-GCaMP6F/AAV-Flex-GCaMP6s), in response to optical stimulation of mPFC axonal terminals expressing channelrhodopsin (AAV-oChiEF-tdTomato). (**A**) Dual-channel, two-photon laser scanning images of LHb neurons expressing GCaMP6F/Flex-GCaMP6s and mPFC axonal terminals in the LHb expressing oChiEF-tdTomato. Overlapping expression is consistent with mPFC axons making synapses onto LHb neurons. (**B**) LHb neuron activity was imaged for 20 cycles with 25 frames in each cycle. Average LHb neuron activity at each frame for all 20 cycles was plotted (black line), with peak response occurring at the 13th frame when mPFC synapses were stimulated. LHb neuron activity was recorded for 3 experimental conditions: before NE addition ('baseline'), after NE addition (NE (5μM)), and after NE removal ('wash'). (**C**) Summary of normalized LHb neuron activity responses to each experimental condition (mean ± SE). Addition of NE significantly potentiated LHb neuron activity response to stimulation of mPFC axonal terminals (paired t-test: n=17, t=1.76, *p=0.011). This synaptic potentiation effect by NE included selective mPFC inputs to VTA projecting (paired t-test: n=10, t=1.83, *p=0.040) and RMTg projecting LHb neurons (paired t-test: n=10, t=1.83, *p=0.045).

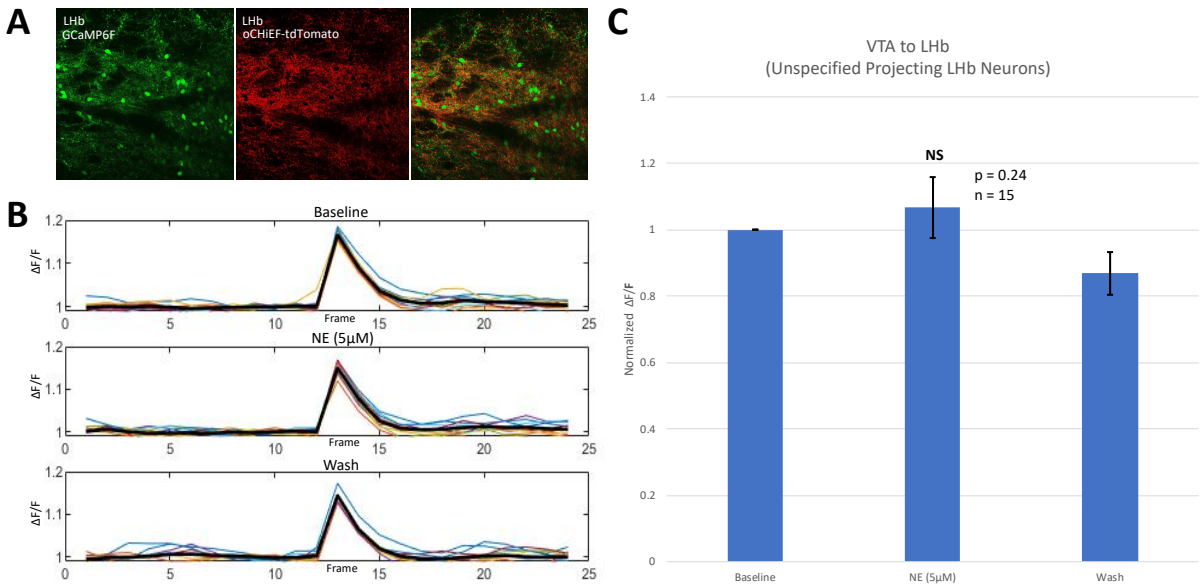

Figure 3. NE does not potentiate VTA inputs to the LHb. VTA axonal terminals in the LHb expressing channelrhodopsin (AAV-oChiEF-tdTomato) were photostimulated and response of LHb neurons expressing a calcium indicator (AAV-GCaMP6F) were imaged using a two-photon laser scanning microscope. (**A**) Dual channel, two-photon laser scanning images of LHb neuron cell bodies expressing GCaMP6F and VTA axons in the LHb expressing oChiEF-tdTomato. Overlapping expression supports innervation of LHb neurons by VTA axonal terminals (**B**) Recordings as in Figure 2. (**C**). Summary of normalized LHb neuron activity response to each experimental condition (mean ± SE). Addition of NE does not significantly potentiate VTA inputs to the LHb (paired t-test: n=15, t=1.76, p=0.24).

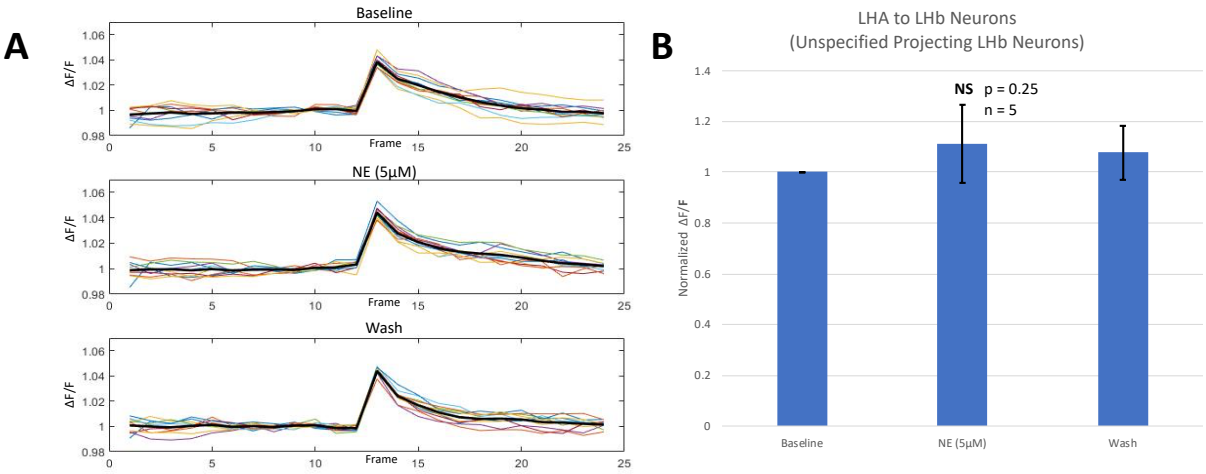

Figure 4. NE does potentiate LHA inputs to the LHb. LHA axonal terminals in the LHb expressing channelrhodopsin (AAV-oChiEF-tdTomato) were stimulated and response of LHb neurons expressing a calcium indicator (AAV-GCaMP6F) were imaged using a two-photon laser. (**A**) Recordings as in Figure 2. (**B**) Summary of normalized LHb neuron responses to each experimental condition (mean ± SE) indicates no significant potentiation of LHA inputs to the LHb (paired t-test: n=5, t=2.13, p=0.25) was observed by the addition of NE.

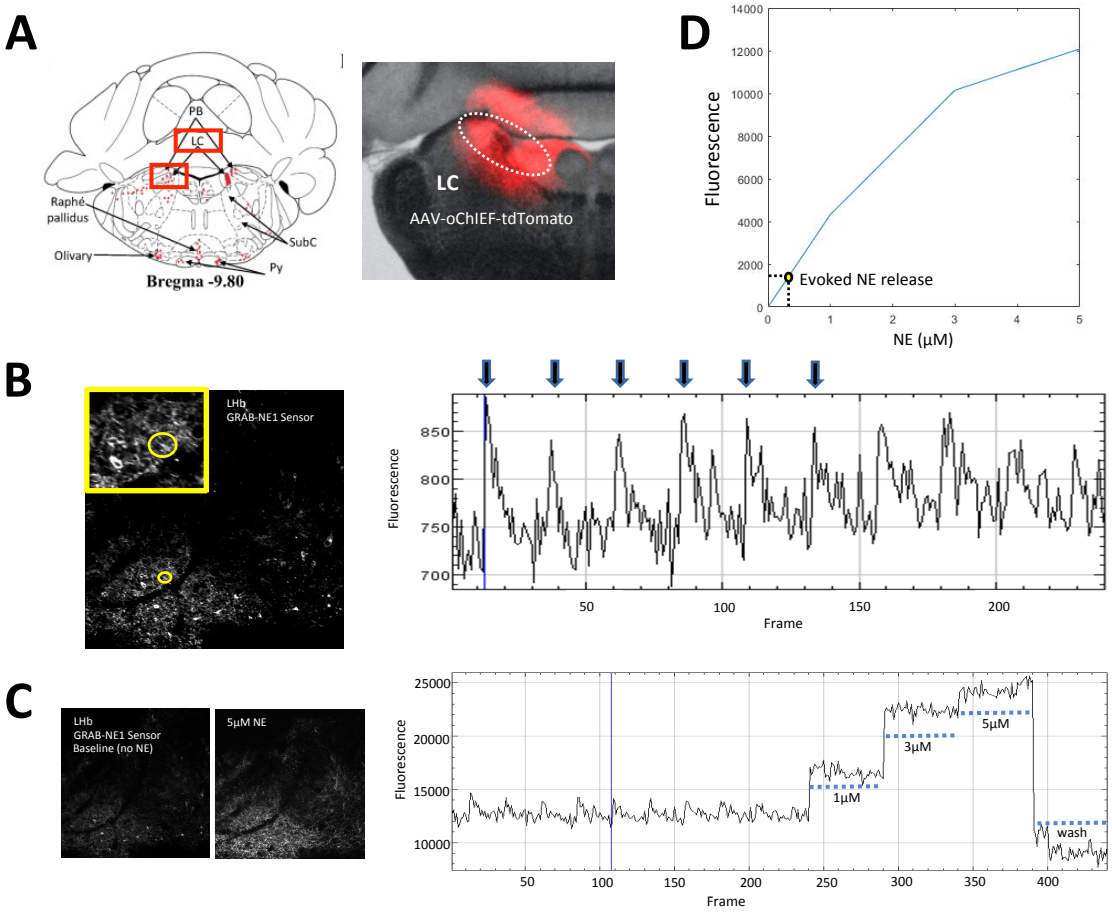

Figure 5. *Spontaneous and evoked NE release in the LHb from Locus Coeruleous terminals.* A NE sensor (AAV-GRAB$_{NE1}$) was injected into the LHb and channelrhodopsin (AAV-oChIEF-tdTomato) into the locus coeruleus (LC) for two-photon calcium imaging and optogenetics. (**A**) A dissection microscope image showing accurate injection site of channelrhodopsin into the LC. (**B**) Spontaneous NE release was identified onto a LHb neuron (circled) and NE release could be evoked by stimulating LC terminals. Arrows indicate time of stimulation and the corresponding spiking increase in fluorescence from the sensor detecting increases in NE concentration. (**C**) Different concentrations of NE (1μM, 3μM, 5μM) was added to test the sensitivity of the sensor and to confirm that the sensor was responding to increases in NE concentration. (**D**) The concentration of NE was plotted with its corresponding fluorescence intensity and the concentration of evoked NE release in LHb was estimated to be < 0.05μM.

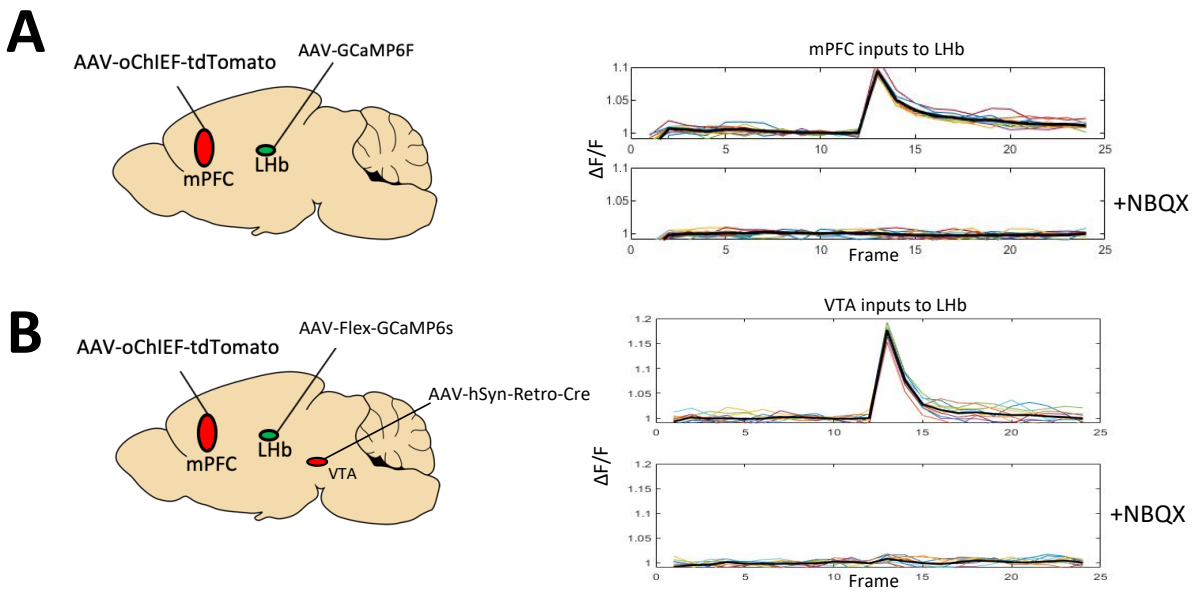

Figure 6. Both mPFC and VTA inputs to LHb are excitatory and glutamatergic. (**A, B**) Calcium imaging data show increase in fluorescence when mPFC and VTA terminals in the LHb were stimulated, indicating excitatory response of LHb to both mPFC and VTA input. Both mPFC and VTA projections are glutamatergic, as addition of NBQX eliminated excitatory responses.

Table 1. Raw calcium imaging data (dF/F). Responses of LHb to stimulation of mPFC, VTA, and LHA terminals in the LHb. 5 slice recordings from each synaptic input experiment are shown. The baseline value displayed is the average of two baseline recordings taken for each slice. Compared to VTA and LHA inputs, mPFC inputs to LHb are relatively weak but are potentiated by the addition of NE.

	Baseline	**NE (5µM)**	**Wash**
mPFC to LHb	0.0065	0.015	0.0121
	0.001025	0.0032	0.00145
	0.0061	0.01895	0.0044
	0.0093	0.00945	0.00365
	0.003675	0.00825	0.00485
VTA to LHb	0.0622	0.0695	0.0365
	0.01405	0.019	0.0135
	0.0133	0.015	0.0194
	0.03625	0.0375	0.0268
	0.012175	0.0135	0.0127
LHA to LHb	0.12675	0.0738	0.096
	0.07045	0.10835	0.07065
	0.0376	0.04165	0.0375
	0.037275	0.0415	0.048
	0.021525	0.02625	0.0288

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