

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Entopeduncular projections to the lateral habenula are excitatory and behaviorally
aversive in rodents

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by

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Chair

University of California, San Diego

2012

DEDICATION

For my family: for the love and support they've given me, for letting me pursue my goals, and for putting up with my bad financial skills.

For Bib: who gave me the encouragement to keep going. And for waking me up on time.

For my friends.

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The methods section is, in part, taken from a paper that is in publication. Dr. Steven Shabel, Dr. Christophe Proulx, Ryan Murphy, myself, and Dr. Robert Malinow are co-authors. Dr. Steven Shabel and Dr. Christophe Proulx were the primary investigators.

The results section is, in part, taken from a paper that is in publication. Dr. Steven Shabel, Dr. Christophe Proulx, Ryan Murphy, myself, and Dr. Robert are co-authors. Dr. Steven Shabel and Dr. Christophe Proulx were the primary investigators.

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ABSTRACT OF THE THESIS

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Professor Robert Malinow, Chair

The lateral habenula has recently been implicated in encoding anti-reward signals during moments of disappointment. Specifically, the lateral habenula has been shown to inhibit dopamine neurons in the reward system. However, its relationship to the basal ganglia, a major input to the lateral habenula, remains unclear. Recent models have proposed that the basal ganglia may serve as the driving force behind increased habenular activity despite the basal ganglia's primarily inhibitory nature. Here, we report that these inputs to the lateral habenula are, in fact, excitatory and behaviorally aversive. Moreover, we also show that serotonin, a neuromodulator associated with depression,

presynaptically modifies inputs from the globus pallidus. However, more research is needed for higher resolution of the synaptic and behavioral aspects of basal ganglia input.

Introduction:

Mood disorders are a significant medical concern in the Western world, affecting approximately 15% of the population across the US and Western Europe (Cassano et al., 2002). Major depressive disorder (MDD) and bipolar disorder are among these mood disorders that can be further categorized into subtypes. Some psychological criteria used to characterize MDD include depression, irritability, reduced concentration, and low self-esteem; unfortunately, current experimental measures tested on animals cannot quantify these traits without attributing anthropomorphic errors (Cassano et al., 2002). However, other defining characteristics of MDD also include anhedonia (a loss of pleasure from things that were once pleasurable) and lack of motivation (Cassano et al., 2002). These two particular features are of note since they can be measured and used quantifiably, forming an analog to the rodent model of depression that uses immobility as a measure. A particular region of interest associated with depression is the lateral habenula (LHb), located in the posterior-dorsal-medial region of the epithalamus.

The lateral habenula functions mainly as a relay between the forebrain and brainstem. Recent studies indicated that the habenula plays a multifunctional role in behavior by modulating sleep, motor control, decision making, and responses to stress, anxiety, and reward (Hikosaka, 2010). In particular, the LHb's responses to stress, anxiety, and reward have been viewed as corollaries in studies linking the LHb to MDD. In rodents exhibiting depressive behaviors, lesioning of the LHb can restore normal behavior (Hikosaka, 2010). Other studies have indicated that deep brain stimulation, used to treat severe depression also alleviates depressive symptoms (Li, 2011).

Currently, there have been studies examining the LHb's newfound role in reward seeking behavior. Studies on the habenula observed neuronal firing patterns and synaptic characteristics to elucidate the information encoded by the circuit in reward based tasks in animals. These experiments have shown a decrease in dopaminergic neuron activity during simultaneous increase in habenula activity (Hikosaka, 2010). Dopaminergic pathways such as the ventral tegmental area (VTA), substantia nigra (SNc), and nucleus accumbens (NAc) are reported to be regions associated in reward seeking behavior, motivation, and are also LHb efferents (Hikosaka, 2010). Matsumoto and Hikosaka (2008) have verified that decreased activity within dopaminergic pathways follows an increase lateral habenula activity, suggesting that the LHb functionally inhibits these nuclei. SNc dopaminergic neurons and LHb neurons of rhesus monkeys were simultaneously recorded while the animals performed a visual task (Matsumoto et al., 2007). The visual task is associated with reward, and after a number of successful trials with reward, the task is then paired with no reward. The authors observed that while both LHb and SNc neurons responded to these tasks, they responded in opposite manners. SNc neurons showed increased activity during reward paired tasks and decreased activity during no reward tasks while the opposite occurred in LHb neurons.

In humans, research has implicated the role of serotonin (5HT) in modulating the LHb in individuals who suffer from MDD through the use of positron emission tomography (PET). In one study, individuals suffering from MDD were given verbal task to perform while the PET scan was in progress (Morris et al., 1999). To test for 5HT's role in modulating habenula activity, subjects were placed on a low tryptophan

diet, a precursor to 5HT, and on test day were either given a Tryptophan-balanced drink or a tryptophan-depleted drink before the trial (Morris et al, 1999). During the testing period, PET scans indicated a significant increase in LHb and dorsal raphe nucleus (DRN) activity in those subjects treated with the tryptophan-depleted treatment (Morris et al., 1999). The results suggest that 5HT may play a significant role in alleviating depressive behavior, particularly in the LHb.

Surprisingly, while there are numerous studies on the LHb and its role in behavior, fewer studies have been done on the globus pallidus (GP), the entopeduncular nucleus (EP) in non-primates. Herkenham et al. (1977) showed that approximately 60% of the LHb receives input from the entopeduncular nucleus. Localized injections of horseradish peroxidase (HRP), acting as a retrograde tracer, into the LHb were able to show afferent connections to the LHb which mainly stemmed from a distinct region in the EP (Herkenham et al., 1977). Therefore, EP inputs are a major source of input to the LHb. Previous findings have characterized the GP/EP as a primarily inhibitory nucleus. In one particular experiment, HRP and GABA-T histochemistry showed that projections to the LHb contain a significant amount of cells doubly stained for HRP and GABA-T within EP nuclei (Araki et al., 1983). In another study, inhibitory markers were studied in the EP after ablation of the LHb; results indicated decreased levels of GABA markers in the LHb after EP lesioning (Penney & Young, 1980). More interestingly, Araki and colleagues also noted that not all projections to the LHb were GABAergic, indicating that 60% of these EP projections were not doubly stained for GABA-T (Araki et al., 1983). Thus, projections to the LHb may not consist solely of inhibitory neurons.

New research has characterized the functionality of GP projections suggesting that a majority of these connections respond to incidents of disappointment, akin to the LHb (Hong & Hikosaka, 2008). Increased firing rates in this region occur prior to increases in habenula activity. Therefore, it seems that a functional discrepancy exists between EP function and the LHb since EP function to this point has been viewed as inhibitory. Taken together, data suggest that the EP may also contain excitatory neurons which drive habenular activity. Hong and Hikosaka have proposed a model for this pallidothalamic circuit, positioning the GP upstream of the LHb, and have also identified two functionally distinct outputs from the GP. Observations have shown that the two types of outputs from the GP project towards either the LHb or the thalamus for either reward evaluation or motor control, respectively. As a whole, these data conflict with the classical inhibitory role of the GP.

The purpose of this study is to identify and characterize the neurons which project from the EP to the lateral habenula. In doing so, we hope to gain a better understanding of the relationship of the EP to the LHb, whether it is an excitatory or inhibitory relationship. To do this, whole-cell recordings were performed on habenula neurons while stimulating presynaptic EP inputs. We injected an adeno-associated virus (AAV) expressing channelrhodopsin-2 (ChR2) into the EP of rodents to provide presynaptic stimulation of the LHb. ChR2 functions as a cation channel when optically stimulated thus allowing for selective initiation of action potentials. By treating neurons with NBQX, a glutamate receptor antagonist, we can quantify the excitatory or inhibitory nature of these terminals by studying the currents elicited at these synapses.

Furthermore, immunohistochemistry performed on fixed tissue slices identified the major synaptic terminals to the LHb originating from the EP. Using antibodies staining for GAD67 (GABAergic neurons) and VGLUT2 (glutamatergic neurons), one can observe either an inhibitory or excitatory relationship, respectively.

We also wished to test the effects serotonin on modulating EP inputs to the LHb by measuring the postsynaptic response of habenula neurons treated with serotonin. We performed whole-cell recordings of habenular neurons with application of either serotonin or dopamine and quantified postsynaptic current elicited from EP stimulation. Analysis of paired pulse ratios revealed the modulatory effect of the treatments on presynaptic EP fibers.

Next, assays measuring rodent aversion revealed the behavioral effect of EP stimulation to the LHb. To this end, we injected an AAV expressing ChR2 into the EP of rodents and fitted bilateral optical cannula above the LHb to allow for selective stimulation of EP fibers to the LHb. We then optically stimulated EP projections during the directed place preference (DPP) assay to observe the behavioral effect of EP stimulation. The animals were placed in a two-compartment shuttlebox with free access to both compartments and no optical stimulation. After some time, EP stimulation occurred when the animal was present in one specific compartment and not the other. The amount of time spent in each compartment during EP stimulation allowed for a measure of aversion.

As follow up experiments, we tested the behavioral effects of serotonin in vivo by treating rodents with an SSRI during the DPP behavioral assay. We also tested for

changes in reward detection by attempting to deactivate presynaptic input from the EP through the use of a reactive oxygen species (ROS) fusion protein mSOG. The mSOG protein generates singlet oxygen, $^1\text{O}_2$, ROS which, in high amounts, leads to oxidative damage in cells. mSOG fused to synaptophysin localized oxidative damage to presynaptic vesicles and would then disrupt synaptic transmission. Although we did not find conclusive behavioral results in applications of SSRIs or ROS fusion protein, we speculate that alternative methods may bear more conclusive results.

As a whole, our results indicate a reversible and significant increase in aversive behavior during EP stimulation, characterizing an excitatory relationship between the EP and Lhb in both cellular recording and behavior.

Methods:**Animals:**

Male Sprague-Dawley rats, age 28-32 days at time of surgery (42-46 days when sacrificed for slice experiments), were housed 1-4/cage and kept on a 12/12 hour light-dark cycle (lights on/off at 6 am/6 pm). All procedures involving animals were approved by the Institute Animal Care and Use Committees of the University of California, San Diego.

Virus preparation, injection and cannula implant:

cDNA encoding flexed version of ChR2(H134R)-eYFP was kindly provided by Dr. Karl Deisseroth (Stanford). To make non-flexed version, ChR2(H134R)-eYFP sequence was PCR amplified flanked by KpnI and EcoRI sites and subcloned into pAAV-EF1 α -flexed-ChR2(H134R)-eYFP-WPRE vector using same restriction sites. Sequencing confirmed gene sequence integrity. AAV-EF1 α -ChR2(H134R)-eYFP-WPRE (serotype 1; 9×10^{11} GC ml⁻¹) (named AAV-ChR2-YFP in the text) and AAV-CMV-mCherry were made by Salk Vector Core (La Jolla, CA).

Rats were anesthetized with isoflurane for stereotaxic bilateral injection of AAV-ChR2-YFP into the EP (A-P: -2.1 mm from bregma; M-L: 2.55-2.70 mm; D-V: -6.3 - -6.9 mm from dura). 0.1 – 0.5 μ L of virus were injected into each hemisphere over 8-20 minutes using a picospritzer. Control rats for behavior experiments were injected with an AAV encoding mCherry. The injection pipette was not removed until 10 minutes after the end of the infusion to allow diffusion of the virus. Subjects for the behavioral

experiment were injected with virus as described above and dual fiberoptic cannulae (Doric Lenses, Canada) were implanted in order to have the tip of the fiberoptic cannulae (200 μm , 0.22 NA) above the left and the right LHb (A-P: -3.6 mm from bregma; M-L: +/-0.75 mm; D-V: -4.0 mm from dura) and secured to the skull with screws and dental cement. Rats were injected subcutaneously with 5 mg/kg carprofen (NSAID) after surgery.

Freely moving directed place preference (DPP):

Rats ($n = 7$ in ChR2-YFP group, $n = 5$ in mCherry control group) used for DPP underwent surgery at 4-7 weeks old and behavior experiments were conducted at least three weeks after surgery. DPP was carried out in a shuttle box (50 cm W x 25 cm D x 30 cm H; Coulbourn Instrument) equipped with a door separating the two halves and photocell detectors. Walls were modified in order to present different patterns to provide contextual differences. Photocell detectors allowed automatic monitoring of rat location in the cage for the duration of testing. Optical activation of ChR2-YFP-expressing axons was performed using an optical fiber coupled to a 473 nm solid-state laser diode (OEM laser system, MI) with 20 mW of output from the 200 μm fiber.

Directed place preference was designed in order to monitor preference/aversion induced by optical stimulation of the LHb. Throughout the full duration of the test, rats were free to explore both sides of the cage. The first 10 min allowed us to measure preference for either context without manipulation. No preference was found during this first 10 min. After this 10 min baseline period, optical stimulation (continuous 20Hz, 5 msec pulse duration) was delivered while the animal was in one context (defined as

“context A”). For the next 30 min, optical stimulation of the LHb occurred whenever the rat was located in context A. Optical stimulation was stopped when the animal was in the other side of the cage (context B). Avoidance scores were measured by taking time spent in context B minus time spent in context A divided by total time (120 sec). Student’s T-test compared avoidance score from period 10-40 min to baseline (0-10 min period). In a different set of DPP testing, pairing of the optical stimulation with context A (20 min) was switched to context B for an another 20 min and then paired again with context A for the last 10 min of the 1 hour session (see schematic in **Fig. 3**). One ChR2-YFP-expressing rat lost its cannula before the DPP reversal test, so only 6 ChR2-YFP-expressing rats were tested for reversal of DPP. Student’s T-test compared avoidance score from periods 10-30, 30-50, and 50-60 to baseline period (0-10 min).

Reward-Based Operant Conditioning:

Animals were placed in a two-port oral drug self-administration chamber (Model # ENV-018MD; Med-Associates) and trained to receive reward from either port, each administering equal sucrose reward (10% sucrose in water).

Sucrose delivery was indicated by a light above either port signaling the presence of reward. After 5-6 successful sessions, with 150 total light trials each, rats were then trained to press a lever in order to receive equal reward from either port.

After 5-6 successful lever sessions, rats were then injected with an AAV expressing synaptophysin-mSOG fusion protein (mSOG) into the EP and then fitted with a bilateral cannula. Rats were allowed to recover for 2-3 weeks and then retrained in

lever pressing for 3-4 successful sessions. The animals were connected to the optical equipment prior to each experimental session with alternating sessions of optical stimulation.

Experimental sessions were similar to lever training sessions with the addition of dual-light trials, presenting the animal with both lights illuminated, forcing the animal to choose from either side and delivering equal reward amounts (2 boluses). Dual light trials were interspersed with single illumination trials for either side. 21 dual-light trials were administered allowing for baseline preference. After baseline, 39 dual-light trials were administered giving unequal reward amounts dependent upon their baseline preference. For downshift experiments, dual-light trials administered a lower reward (1 bolus) on the preferred side and normal reward (2 boluses) on the non-preferred side. During upshift experiments, dual light trials administered an increased reward (4 boluses) on the non-preferred side and normal reward (1 bolus) on the preferred side. Shifting the reward in either the downshift or upshift paradigms would effectively trigger unexpected moments of disappointment. The operant based behavior experiment was designed to observe the change in reward detection by the subject. By changing the amount of expected reward, we could observe the rate of the change in preference and the overall side preference during each session.

For double light trials, preference was measured by counting the number of times the subject chose a side and dividing by the total number of trials. Shifts in preference were measured by comparing the mean preference before and after the change in reward. Student's T-test was then used to compare mean preferences before and after the shift.

Fluoxetine & Saline Injections:

Animals were weighed prior to each injection and administered either fluoxetine (diluted in normal saline solution, 2mg/mL) at 5mg/kg of body weight or normal saline solution at 5mg/kg of body weight prior to each DPP session. Intraperitoneal injections were performed using a 27.5 gauge needle. Aspiration was performed upon insertion of needle. Injection sites were cleaned and massaged pre and post injection.

Electrophysiology:

Two weeks after surgery, rats were anesthetized with isoflurane before decapitation and brain removal. Brains were chilled in ice-cold dissection buffer (110.0 mM choline chloride, 25.0 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 0.5 mM CaCl₂, 7.0 mM MgCl₂, 25.0 mM glucose, 11.6 mM ascorbic acid, 3.1 mM pyruvic acid; gassed with 95% O₂/5% CO₂) and cut in 400 micron thick coronal slices through the EP and LHb. Slices were transferred to 35 °C ACSF (118 mM NaCl, 2.5 mM KCl, 26.2 mM NaHCO₃, 1 mM NaH₂PO₄, 20 mM Glucose, 4 mM MgCl₂, 4 mM CaCl₂; 22°–25°C; pH 7.4; gassed with 95% O₂/5% CO₂) for 30 minutes. After an additional 30 minutes of recovery at room temperature, slices were transferred to the recording chamber and constantly perfused with 27 °C ACSF.

Recordings were made from cells in the lateral half of the LHb, where ChR2-YFP expression was highest, using an Axopatch 1D amplifier with a 5kHz sampling frequency and a filter set at a -3dB cutoff frequency of 5kHz. For current-clamp recordings the intracellular solution consisted of (in mM): 130 K-Gluconate, 5 KCl, 10 Hepes, 2.5

MgCl₂, 4 Na₂ATP, 0.4 Na₃GTP, 10 Na-phosphocreatine, 0.6 EGTA (pH 7.2). For voltage-clamp recordings the intracellular solution consisted of (in mM): 7.5 QX314, 115 cesium methanesulfonate, 20 CsCl, 10 HEPES, 2.5 MgCl₂, 4 Na₂-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, and 0.6 EGTA (pH 7.2). 470 nm light pulses were delivered to the LHb via an optic fiber attached to an LED. To examine the effect of serotonin (1 μM in all experiments) on excitability, we also injected current (-100, 0, 100, 200, 300 pA) for 300 ms – one current injection every 10 seconds in current-clamp. To examine the effect of serotonin on light-evoked currents, cells were clamped at -50 mV and 0.5-5 ms light pulses were given in pairs, 100 ms between each pulse, every 10 seconds. Picrotoxin (100 μM) was included in the extracellular recording solution during voltage clamp but not current clamp recordings. For I-V recordings, NBQX (3 μM) and APV (100 μM) were added during the recordings taken at -60, 0, and 40 mV (minimum 10 sweeps taken at each potential before and after each drug) to determine the contribution of AMPA and NMDA receptors to excitatory currents. After recordings were completed, slices were fixed in 4% formaldehyde overnight and mounted on slides for localization of the injection site and labeled projection to the LHb.

Data analysis:

To measure the effects of EP fiber stimulation on membrane potential for each cell, the slope of the postsynaptic potential (PSP) was measured (average of 3 sweeps) at resting potential. The same start and peak times were used to compute the PSP slope during depolarization (average of 3 sweeps). The time intervals used to determine the effect of serotonin on excitability were 0-150 s before serotonin and 7.5-10 min after

serotonin. Excitatory current amplitude was measured as the peak amplitude in a 2 ms window between 3-20 ms after the light pulse.

Statistical analysis:

All values given in the text and figures indicate mean \pm SEM. Student's t test was used with $p < 0.05$ deemed significant. Tukey's HSD was used to compare multiple means to control group mean for operant conditioning, a $p < 0.05$ was deemed significant.

Tracer injection, perfusion and tissue processing:

To label LHb-projecting EP neurons, 0.5 μ l of Cholera toxin subunit B conjugate to the AlexaFluor488 (CTx⁴⁸⁸) (2 mg/ml in phosphate-buffered saline, PBS pH 7.4) was unilaterally injected in the LHb (AP: -3.6 mm, ML: 0.7 mm, DV: -4.8 mm) over 5-7 minutes. Rats were allowed to survive for 40 hours, were perfused and brains processed for immunohistochemistry.

For perfusion, rats were deeply anesthetized using a mix of ketamine/dexdomitor (75 and 5 mg/Kg respectively IP) and transcardially perfused with saline followed by a solution of phosphate buffer 0.1 M (PB, pH 7.4) containing 4 % paraformaldehyde. Brains were postfixed overnight in the same solution, rinsed with PB and cryoprotected by immersion in PB/30% sucrose solution for three days. Frozen brains were sectioned at 50 μ m with a sliding microtome in the coronal plane.

For each brain, 3 slices encompassing the entopeduncular nucleus were chosen for immunohistochemistry. Free floating slices were first blocked in TN (Tris 0.1M, 1%

NaCl, pH 7.4) buffer containing 10% normal goat serum and 0.2% triton X-100 for 3 hours. After blocking, slices were incubated with the following antibodies diluted in TN/3% NGS/0.2% Triton X-100 solution; anti-VGLUT2 (Millipore) or anti-GAD67 (Millipore) for 48 hours at room temperature. After 3 washes in TN buffer, slices were incubated with secondary antibody Alexa Fluor647 goat anti-mouse (Invitrogen) in TN/3% NGS/0.2% Triton X-100 for 4 hours at RT. Slices were washed and mounted using Vectashield mounting medium (Vector Laboratories). Images were taken with a FV1000 confocal microscope (Olympus), adjusted for brightness using Fluoview software and assembled in Adobe Illustrator.

The methods section is, in part, taken from a paper that is in publication. Dr. Steven Shabel, Dr. Christophe Proulx, Ryan Murphy, myself, and Dr. Robert Malinow are co-authors. Dr. Steven Shabel and Dr. Christophe Proulx were the primary investigators.

Results:

Immunohistochemistry indicates that EP inputs to the LHb are both Glutamatergic and GABAergic

Previous histochemical studies have shown the presence of GABAergic neurons in the pallidal nuclei (Araki et al., 1984; Penney & Young, 1981). However, recent findings have suggested that the neuronal population within the EP projecting to the LHb also contains glutamatergic neurons (Barroso-Chinea et al., 2008). To confirm this finding, we injected retrograde tracer cholera toxin tagged with Alexa 488 (CTx⁴⁸⁸) into the LHb to identify EP soma inputs (Fig. 1a). Next, immunohistochemistry was performed using VGLUT2 and GAD67 to label glutamatergic and GABAergic cells, respectively (Fig. 1b). Quantification of cells expressing GAD67 or VGLUT2 suggested that approximately 2/3, or 67%, of the EP population were positive for VGLUT2 (Fig. 1b). These data indicate that LHb-projecting EP neurons do indeed express glutamate.

Electrophysiology indicates that EP inputs to the LHb are predominantly excitatory

Previously, studies indicated that the EP was an important inhibitory nucleus projecting to areas in and around the thalamus (Parent et al., 2001; Oertel et al., 1984). However, new findings suggest the presence of an excitatory connection from the EP to the LHb (Barroso-Chinea et al., 2008; Hong & Hikosaka, 2008). Barroso-Chinea and colleagues evidenced the presence of VGLUT2 mRNA in pallidal neurons in rodents, suggesting that pallidothalamic connections may contain excitatory synapses. Hong & Hikosaka have shown that EP spike firing rates are similar and occur prior to habenular firing patterns during moments of disappointment. These experiments collectively

suggest that the EP-LHb circuitry is regulated with the use of excitatory inputs from the EP, in contrast to the classical inhibitory nature of EP. In this experiment, we show that synaptic inputs from the EP can be glutamatergic.

Male Sprague Dawley rats were injected with adeno-associated virus (AAV) expressing Channelrhodopsin2 conjugated to yellow fluorescent protein (ChR2-YFP) into the EP of these rats. Two weeks later, coronal slices were prepared for electrophysiology. These slices showed localized expression of ChR2 in the EP and projections to the LHb (Fig 2a, b). To test for excitatory EP inputs to the LHb, whole-cell recordings were performed on LHb neurons using 470 nm optical stimulation over these EP inputs to stimulate action potentials, postsynaptic potentials (PSPs) in LHb neurons showed depolarization after optical stimulation of these inputs (Fig. 2c left). When habenula neurons were under voltage clamp, optical stimulation of EP inputs again showed positive PSPs (Fig. 2c right). Application of NBQX, a glutamate receptor antagonist, during recordings eliminated these excitatory PSPs seen in the LHb neurons (Fig. 2d, e). To characterize the specific receptors mediating this excitatory response, APV or NBQX was used in cellular recordings to differentiate between NMDA and AMPA receptors, respectively. Results showed that while LHb neurons were held at increasingly depolarized potentials, AMPA currents generally showed a larger inward-rectifying current at each step (Fig. 2g). With these data together, we report that EP inputs to the LHb are largely excitatory and mediated by AMPA receptors, in congruence with Hong & Hikosaka's proposed circuit model. However, exactly how these inputs manifest behaviorally was still unclear at the time.

Stimulation of EP inputs to the LHb is aversive in a single context

As mentioned, the LHb has been linked to the depression and reward seeking behavior. Increases in habenular activity occur during instances of anti-reward outcomes, that is when an expected reward is not received, and are encoded as negative outcomes (Matsumoto & Hikosaka, 2007). As the EP comprises approximately 60% of habenula inputs (Herkenham & Nauta, 1977), activation of these axons may very well drive habenula activity during these negative outcomes. To observe the behavioral effect of stimulating EP-LHb projections, *in vivo* stimulation of these axons was conducted during DPP to measure context avoidance in rodents.

Rodents were injected with either AAV expressing Chr2-YFP or AAV expressing mCherry into the EP, then fitted with a bilateral optic fiber cannula over the LHb. Two weeks later, animals were then placed inside a two-room shuttle box allowing for free access to either side, or context. Each context contained different wall patterns to allow for discrimination between contexts. Baseline measurements from both the animals expressing Chr2 or mCherry indicated minimal preference for either context during the initial phase of DPP (Fig. 4a, b). However during context conditioning, when optical stimulation is contingent upon location, animals expressing Chr2 showed a marked preference for the context which did not deliver activation of Chr2 compared to baseline (Fig. 4a,b; Chr2-YFP $n = 7$, $*p < .05$). Control animals did not indicate any preference for either context during context conditioning, indicating no aversion to the light (Fig. 4a, b; mCherry $n = 5$, $p > .05$). In sum, activation of these EP inputs to the LHb was sufficient in eliciting an aversive response which is in agreement with the anti-reward

encoding functionality reported by Hikosaka and colleagues. However, no reward-predicting stimuli were necessary for our behavioral assay.

Stimulation of EP inputs to the LHb is effective in reversing a previously learned behavior

Initial sessions of DPP utilized a single context to which the animal would be exposed to optical stimulation. In the latter sessions, the assay was modified by adding context reversals which would elicit optical stimulation in the opposite context. Therefore if EP inputs are aversive, shifting light delivery to opposite contexts should drive a change in preference. In Fig. 5a and c, habituation again did not indicate considerable preference for either side in both mCherry or ChR2 expressing animals. In mCherry expressing animals, during conditioning 2 and conditioning 3, the initial preference observed in the previous respective phase was unaffected by the different phases of conditioning (Fig 5a, b; mCherry $n = 5$, $p > .05$). However, in ChR2-YFP expressing animals, we observed significant shifts in avoidance scores across all 3 phases of conditioning compared to baseline (Fig. 5 c, d; ChR2-YFP $n = 7$, $*p < .05$, $**p < .01$). Here, we demonstrate that stimulation of these EP inputs is not only aversive but also able to reverse contextual preference from a previously learned behavior. The data also agree with our previous finding that light delivery alone is not aversive.

Incidental stimulation of inputs peripheral to the LHb did not elicit significant preference/aversion

EP efferents project not only to the LHb but to other regions of the brain as well, particularly the thalamus. In Fig. 5, tissue slices showing optic fiber terminals and

expression of ChR2 revealed minimal expression in these proximal thalamic regions. Possible activation of these thalamic inputs proximal to the LHb may inadvertently occur during optical stimulation and affect behavior. However, the estimated effective stimulation region does not suggest that regions peripheral to the LHb were activated. To address this, we used preference score as an indicator of performance, with good performance equaling large differences in avoidance scores across the three conditioning epochs. Next, through imaging analysis we observed the optic fiber terminals in tissue slices to approximate the regions of activation when optical stimulation was active. Fig. 6 shows the fiber optic terminals and performance levels of the best and worst animals expressing ChR2. In the worst performing animal, fiber optic terminals were actually found within the anterior region of the LHb, effectively stimulating only the region ventral and peripheral to the LHb in the thalamus. With these observations, we noted that stimulation of the thalamus, but not the LHb, did not elicit aversion in ChR2-YFP expressing animals.

Serotonin modulates input from the EP to the LHb

Previous studies have implicated serotonin in modulating mood disorders and in particular have been well established in depression (Morris et al., 1999). In this experiment, we wished to test the effects of serotonin on the excitability of neurons in the lateral habenula. We then recorded from cells within the LHb receiving inputs from ChR2-YFP expressing EP neurons using paired pulse stimulation (100ms separation) under voltage clamp, applying either serotonin or dopamine during cell recording. We observed a reduction in postsynaptic current after application of serotonin (Fig. 7a; $27 \pm$

6% depression after first light pulse; $p = .001$, $n = 10$ cells) but not with dopamine (Fig. 7a; $7 \pm 5\%$; $p = .26$, $n = 7$ cells). Paired pulse ratios from application of serotonin increased, indicating a reduction in neurotransmitter release and a presynaptic change (Fig. 7b, c; $22 \pm 9\%$ increase; $p = 0.02$). Applying depolarizing current to LHb neurons, we observed no significant change in resting membrane potential between baseline and treatment; thus serotonin did not affect postsynaptic response (Fig. 7d, e; all $p > 0.1$, $n = 13$ cells). Our findings collectively suggest that serotonin provides presynaptic inhibition to excitatory inputs from the EP to the LHb.

Testing the role of serotonin in SSRIs in aversive context conditioning

We have shown that stimulation of EP inputs to the LHb is excitatory and aversive, and also that application of serotonin presynaptically inhibits these inputs. However, it is unclear how this modulation would affect behavior. In order to test for serotonin's effect on context aversion, we performed IP injections on ChR2-YFP expressing rats immediately prior to DPP using either Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), or saline. Following injection, rats were immediately tested in the DPP behavioral assay. Animals were given two separate trials of each treatment with injections prior to all trials. Both treatments allowed the rats to perform the DPP behavioral task similarly to sessions without injection. However, we did not find a significant change in avoidance scores between rats injected with saline or fluoxetine (Fig. 8a,b; ChR2-YFP $n = 5$; $p > 0.05$). We still report the mean behavioral scores for each group since it is possible that chronic treatment with fluoxetine may yet yield conclusive results. Alternative SSRIs, such as sertraline or fluvoxamine, may prove to be

more effective as previous studies have observed sertraline to be more effective in modulating habenula activity (Freo et al., 2007). Furthermore, application of serotonin also caused the rats to become lethargic, a possible confound which may be addressed through alternative application of SSRIs (e.g. oral application) or different drugs. Complementary behavioral assays may also be more relevant in observing effects of SSRIs. Tail pinch or foot shock assays have shown to induce cFos expression, a known neural activity marker, in the LHb. However, it is unknown whether or not EP inputs are responsible for eliciting this increase in habenular activity during these behavioral tasks (Smith et al., 1997).

Testing the inactivation of EP inputs using a ROS fusion protein mSOG

In collaboration with the Tsien laboratory, we obtained an optically activated reactive oxygen species (ROS) fusion protein mSOG. The mSOG protein is fused to synaptophysin, a protein associated with presynaptic vesicle release, allowing for localization at the synaptic terminal. Optical activation of mSOG causes oxidative damage leading to a reduction in synaptic transmission.

Studies using operant conditioning in monkeys indicated that EP neurons exhibit differential firing patterns during anti-reward moments (Hong & Hikosaka, 2008). Cell recordings indicated that the EP efferents to the LHb may encode reward predicting information in monkeys trained to predict reward given specific visual cues. The study found that EP firing patterns preceded habenular activity and decreased in frequency while habenular firing patterns increased. We posited that disrupting EP innervation may modulate reward detection.

To identify behavioral effects of modulating excitatory EP inputs, rodents were tested in reward-based operant conditioning. Rats were first trained to press a lever to receive equal sucrose reward from two ports in an operant chamber. After successful training, rats were then injected with the AAV expressing mSOG or AAV expressing mCherry and then fitted with cannulae. In the experimental trial, reward amounts for the preferred port would shift in-session, either decreasing or increasing (Fig. 9a).

Alternating sessions with either optical stimulation or no stimulation were performed prior to each experimental trial. Here, we report the mean preference scores for the upshift and downshift protocols after stimulation and no stimulation (Fig. 9b, c). We did not find significant difference between the control group and mSOG expressing group in any treatment (Fig. 9b, c; $p \gg 0.05$, mSOG $n = 5$, control $n = 2$). However, further testing with more significant suppression of glutamatergic transmission may yield more informative results.

The results section is, in part, taken from a paper that is in publication. Dr. Steven Shabel, Dr. Christophe Proulx, Ryan Murphy, myself, and Dr. Robert are co-authors. Dr. Steven Shabel and Dr. Christophe Proulx were the primary investigators.

Discussion:

Results from our experiments now establish EP inputs to the LHb as excitatory. Consistent with models proposed by Hikosaka and colleagues, our results confirm that excitatory EP inputs drive habenular responses in reward-seeking behavior. We have also confirmed the presence of both glutamatergic and GABAergic neurons within the EP (Barroso-Chinea et al., 2008; Vincent et al., 1982). While we have indicated that EP inputs are dominantly excitatory, we were also able to detect a minimal amount of GABAergic current from the EP. However, we were not able to determine the function of these inhibitory projections in cell recordings or in vivo using SSRIs or by modulating synaptic input through mSOG. We also demonstrate that in vivo stimulation of EP inputs is aversive. Results from behavioral assays show that stimulation of EP fibers produced reversible, contextual aversion. Taken together, these data firmly indicate that EP projections drive aversive behavior and habenula activity.

We also report the effect of serotonin on EP inputs to the LHb. Application of serotonin in cell recordings led to considerable reduction in post-synaptic current and not resting membrane potential in habenula neurons. Furthermore, the change in paired pulse ratio after application of serotonin indicated presynaptic change. However, using fluoxetine during DPP, mimicking the application of serotonin in tissue slices, did not yield significant changes in behavior. We posit that further behavioral testing is required using alternative SSRIs such as sertraline or fluvoxamine as these particular drugs have shown significant effect on the habenula (Freo et al., 2008). Specifically, the authors noted a decrease in habenular activity observed through regional cerebral metabolic rates

for glucose (rCMRglc) autoradiography. While using sertraline and fluvoxamine, the authors detected significantly lower rCMRglc levels with dosage concentrations approximately equal to ours (5mg/kg). However, the authors also noted that to reach similar habenular rCMRglc levels with fluoxetine, considerably higher dosages were needed (40mg/kg). Furthermore, the authors also observed decreases in heart rate and blood pressure which may predict the sluggish behavior found in our assay.

Identifying the function of GABAergic neurons in the EP by suppressing input to the habenula has yielded inconclusive results. Slice preparations indicated that glutamatergic transmission was decreased approximately 10% by using mSOG (data not shown). Altering reward seeking behavior may require more significant suppression of synaptic transmission. Therefore, our findings propose that a more significant reduction in synaptic input is needed for behavioral modification. To this end, inhibiting all synaptic input from the EP through careful lesioning may reveal more conclusive outcomes. Treating neurons with light activated chloride pump halorhodopsin (eNpHR3.0) AAV, rather than ChR2, will cause hyperpolarization in the cells and effectively inhibit synaptic transmission. However, one caveat to this method is that use of halorhodopsin, in the AAV construct on hand, will not selectively target glutamatergic neurons, rather all within the injection site. Thus, it may be possible to target glutamatergic specific cells by driving virus expression through a VGLUT2 specific promoter.

Our findings confirm the neural model proposed by Hong and Hikosaka; that the EP provides excitatory input to the lateral habenula during anti-reward protocols. In this

case, we also show that this pathway is also able to elicit aversive behavior without the need for reward/punishment predicting stimuli. It would seem that EP stimulation within one context is sufficient to drive a significant and reversible shift in preference.

Furthermore, we confirm the presence of GABAergic and glutamatergic neurons within the EP, in contrast to the long held view that the GP is primarily inhibitory. Lastly, we also show that EP projections are modulated by serotonin. In particular, individuals suffering from depression display hyperactive habenular activity, therefore this finding may provide another possible mechanism clarifying methods of action of SSRIs in patients who suffer from depression.

Figures

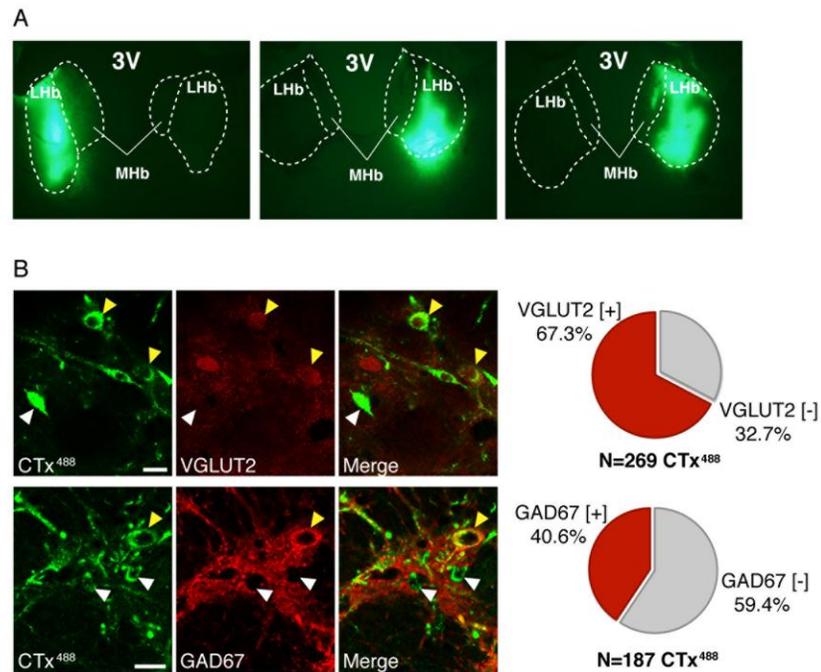


Figure 1: Immunostaining of entopeduncular inputs reveals both glutamatergic and GABAergic neurons. Cholera toxin conjugated to Alexa 488, in green, expressed bilaterally in the LHb from stereotaxic injection (Fig. 1a). Confocal images of cholera toxin stained sections showing nucleus for sections stained either with VGLUT2 or GAD67 antibodies in the EP (Fig. 1b). Pie chart shows percentage of either VGLUT2 expressing or GAD67, red portion, expressing cells (analyzed using Fluoview, Fig. 1b). We observed approximately 67% glutamatergic neurons and 40% GABAergic neurons in the EP. Ctx^{488} used for nuclear staining in the entopeduncular nucleus.

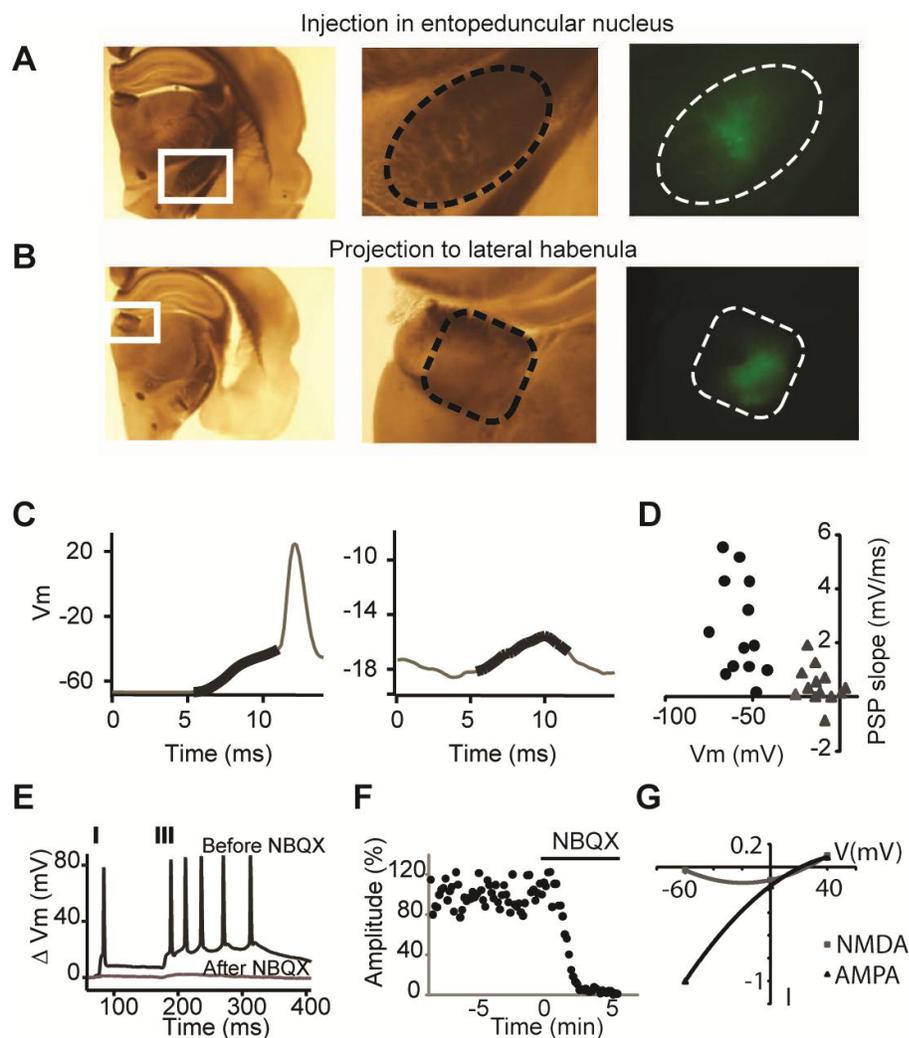


Figure 2: Electrophysiology reveals that entopeduncular input to the lateral habenula is dominantly glutamatergic. Region image of the EP (Fig. 2a) and LHb (Fig. 2b) shows expression of Ctx⁴⁸⁸ and injection into the EP. Electrophysiology was then performed by recording from LHb neurons and stimulation resulted from optic fiber positioned over the EP at 470 nm in 20Hz pulses with 25mW intensity (Fig. 2c-g). Stimulation using light at 470 nm showed positive changes in membrane potential indicating action potentials and functional ChR2 (Fig. 2c left). However, holding the neuron closer to resting potential, optical stimulation still elicited positive membrane voltage changes and inward current (Fig. 2c right, d). Application of NBQX effectively blocked AMPA receptors, showing little/no change in membrane potential or current (Fig. 2e, f). Blocking either NMDA ports or AMPA ports indicate that although both receptors are present postsynaptically, AMPA receptors largely mediate EP inputs (Fig. 2g).

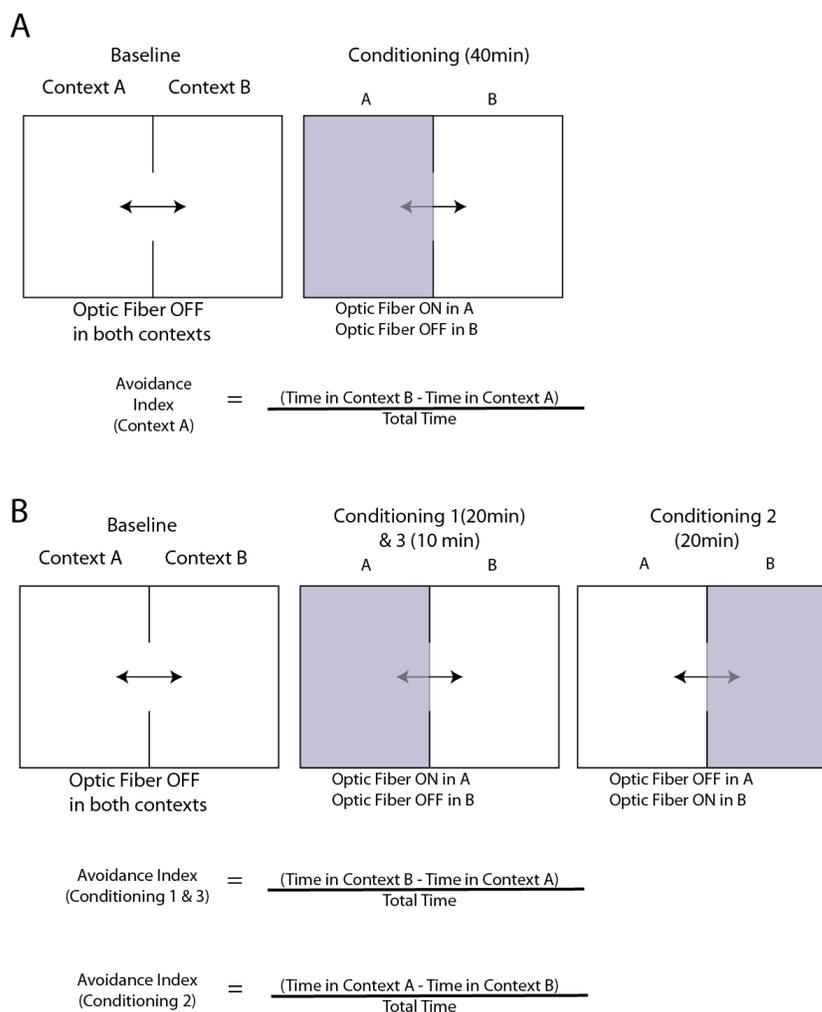


Figure 3: Freely moving directed place preference schematic. Single context and reversal DPP allows the animal to move freely between both sides for the first 10 minutes of baseline without any optical stimulation (Fig. 3a, b). After baseline, context conditioning phase utilizes only one side of the arena to elicit optical stimulation using 470 nm laser, 20mW intensity via a bilateral optic fiber (Fig 3a top). Avoidance index is then calculated to compare baseline preference to context conditioning preference (Fig. 3a bottom). Reversal DPP uses multiple conditioning phases (Conditioning 1, 2, 3) eliciting stimulation during the first conditioning phase on one side, then switching to the other side in the subsequent conditioning phase, then back to the other side on the last phase (Fig. 3b top). Calculations of avoidance index are as before; mean preference scores for each phase were calculated and compared to baseline, respectively (Fig. 3b bottom)

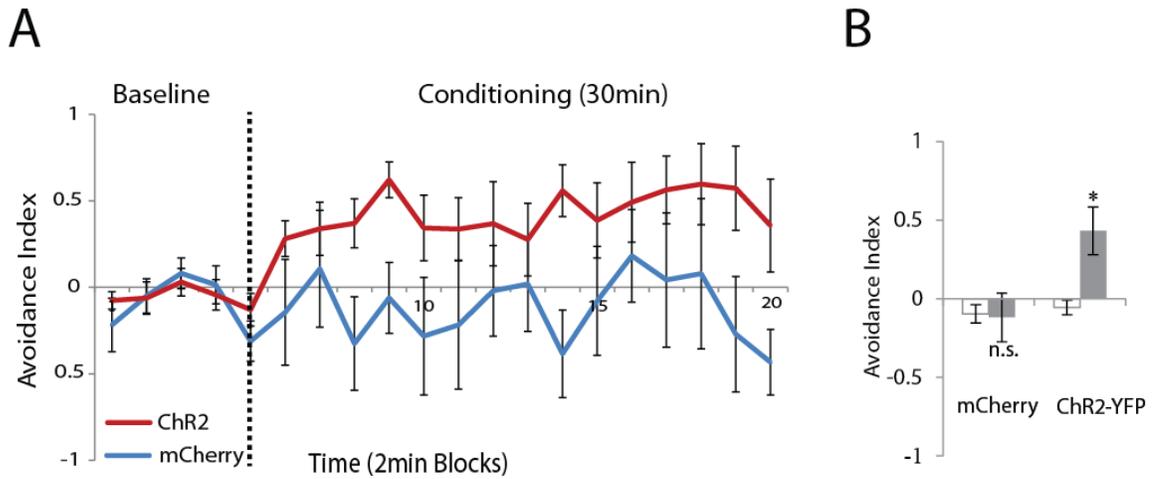


Figure 4: Optical stimulation of entopeduncular inputs is aversive. Figures 4A, B denote the results of single context DPP, blue is the mCherry expressing (control) group and red is the ChR2 expressing group. Figure 4b indicates the mean baseline avoidance index in white and the mean conditioning avoidance index in grey. Figures 4a, b indicate the mean of each experimental group and error bars indicate SEM, t tests were performed comparing mean preference of each phase to the baseline (student's t test was used, $p < 0.05$ deemed significant, ** denotes $p < 0.05$).

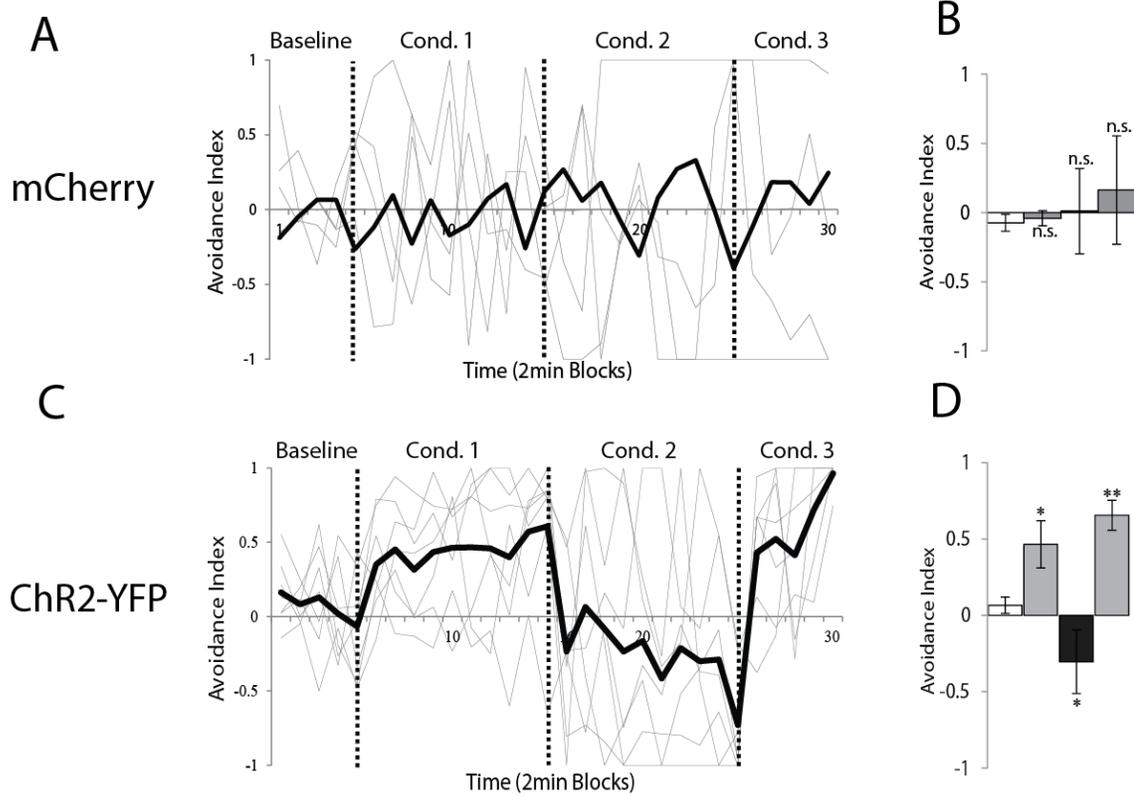


Figure 5: EP stimulation is both reversible and aversive. Figures 5a, b denote the mCherry expressing group mean preference for reversal DPP. Fig 5c and d denote the ChR2 expressing group mean preference for reversal DPP. Light grey lines denote individual subject avoidance throughout the test while the bold black line indicates the mean avoidance score (Fig5 a, c). Figure 5b, d indicate the baseline and individual mean avoidance indexes for each conditioning phase chronologically. Error bars indicate SEM, t tests were performed comparing mean preference of each phase to the baseline (student's t test was used, $p < 0.05$ deemed significant, ** denotes $p \ll 0.05$).

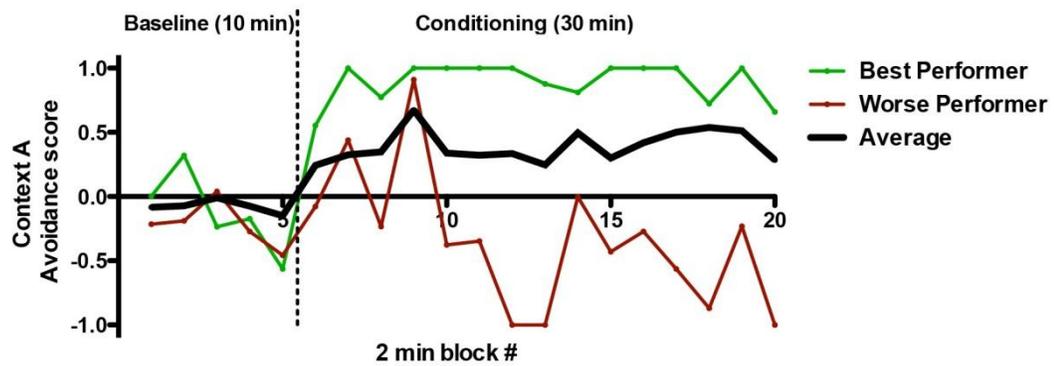
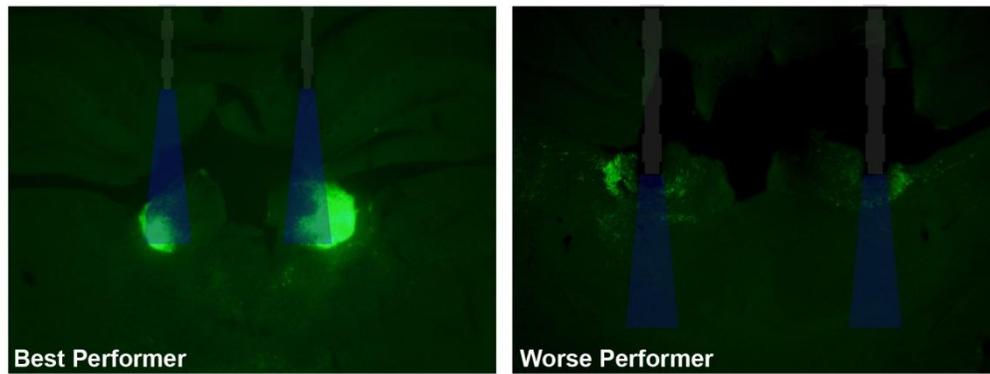


Figure 6: Incidental stimulation of peripheral regions to the lateral habenula is ineffective in eliciting aversion. Fig. 6 shows the best and worst performing rats of the ChR2 group and their respective avoidance scores over time. The worst performer had cannula placed in a lateral aspect of the habenula and the blue cones indicate the calculated effective distance and area the fiber optic could stimulate.

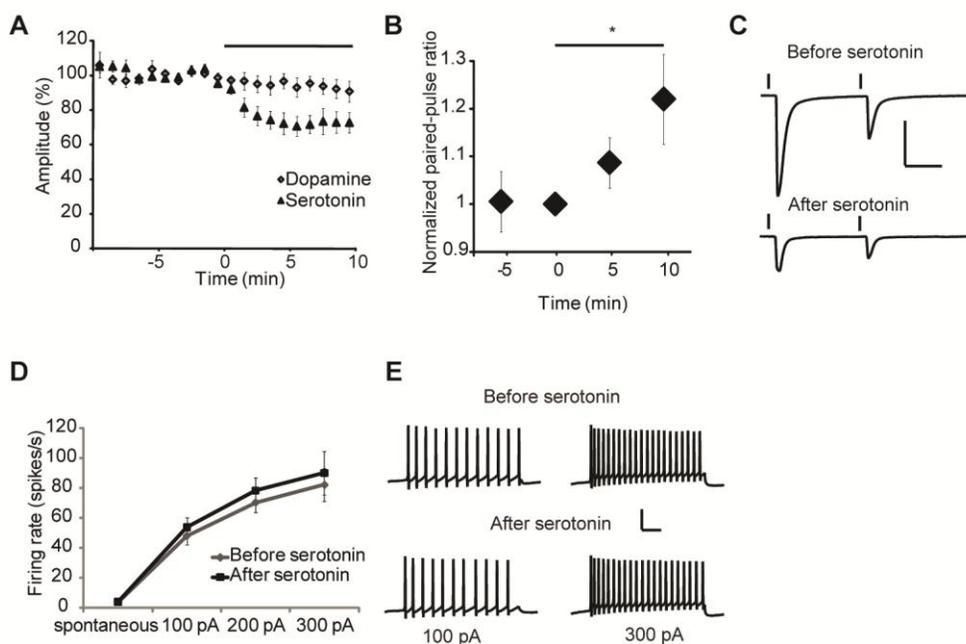


Figure 7: Serotonin presynaptically modulates entopeduncular inputs. We then recorded from cells within the LHb receiving inputs from ChR2-YFP expressing EP neurons using paired pulse stimulation (100ms separation) with clamped voltage, applying either serotonin or dopamine during cell recording. We observed a reduction in postsynaptic current after application of serotonin (Fig. 7a; $27 \pm 6\%$ depression after first light pulse; $p = .001$, $n = 10$ cells) but not with dopamine (Fig. 7a; $7 \pm 5\%$; $p = .26$, $n = 7$ cells). Paired pulse ratios from application of serotonin increased, indicating a reduction in neurotransmitter release (Fig. 7b, c; $22 \pm 9\%$ increase; $p = 0.02$). Applying depolarizing current to LHb neurons, we observed no change in spike firing rate, indicating that serotonin did not affect postsynaptic response (Fig. 7d, e; all $p > 0.1$, $n = 13$ cells).

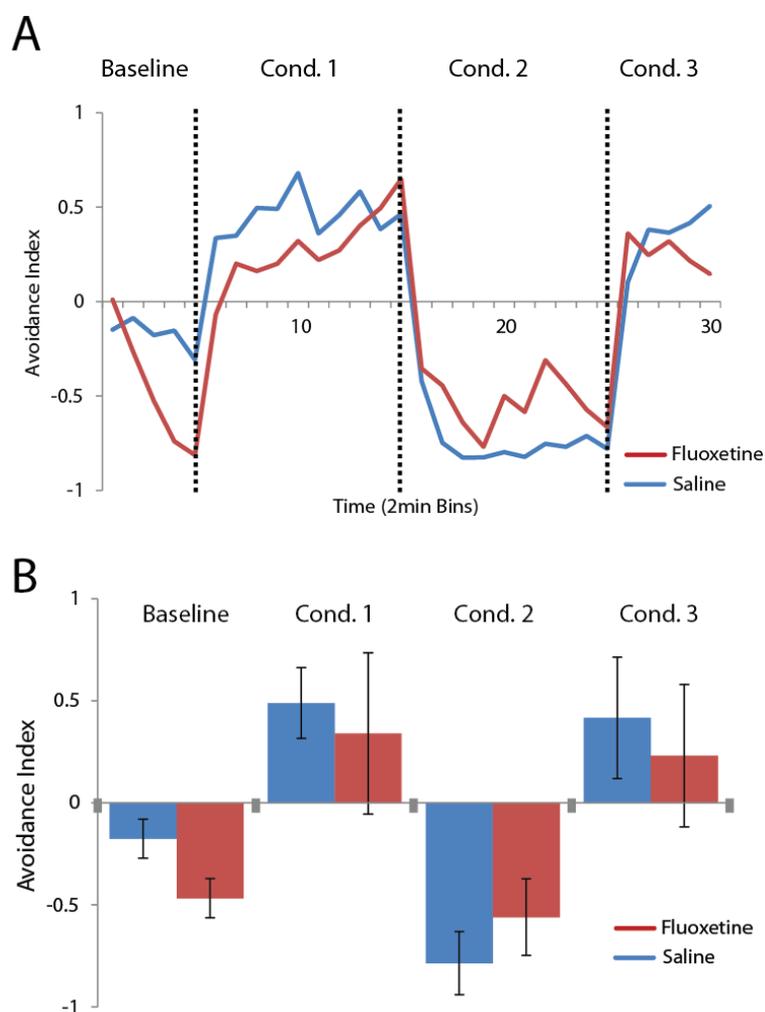


Figure 8: SSRIs may affect entopeduncular input to the lateral habenula. Following injection, rats were immediately tested in the DPP behavioral assay. Animals were given two separate trials of each treatment with injections prior to all trials. Both treatments allowed the rats to perform the DPP behavioral task similarly to sessions without injection, however we did not find a significant change in avoidance scores between rats injected with saline or fluoxetine (Fig. 8a,b; Chr2-YFP $n = 5$; $p > 0.05$). However, we report the mean behavioral scores for each group since it is possible that chronic treatment with fluoxetine may yet yield conclusive results. In both panels, blue denotes the saline injected group while the red indicates the fluoxetine injected group.

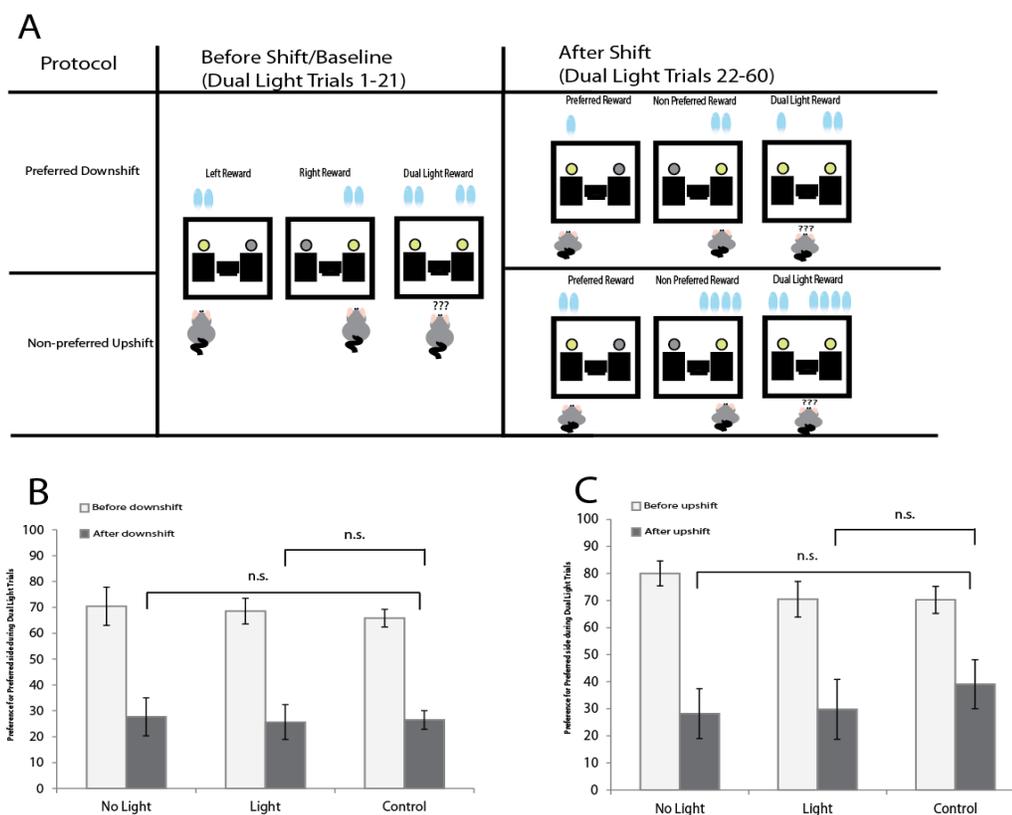


Figure 9: The effect of suppressing EP transmission to the lateral habenula during risk-reward tasks. Different session schedules were taught to the rats with specific levels of reward: Training, Downshift, or upshift (Fig. 9a). Prior to each upshift or downshift session, rats were connected to the optical equipment regardless of whether light activation occurred to maintain similarity throughout the sessions. Here we show the mean preference score \pm SEM for Downshift sessions, we found no significance comparing After Shift means to control mean (Fig. 9b, mSOG $n = 5$, control $n = 2$, $p \gg 0.05$). For upshift sessions, we report the mean preference score \pm SEM, again we did not find significance comparing after shift means to control mean (Fig. 9d, mSOG $n = 5$, control $n = 2$, $p \gg 0.05$). Tukey's test was used to compare After Shift means to control, $p < 0.05$ was deemed significant.

The figures section is, in part, taken from a paper in publication. Dr. Steven Shabel, Dr. Christophe Proulx, Ryan Murphy, myself, and Dr. Robert Malinow are co-authors. Dr. Steven Shabel and Dr. Christophe Proulx were the primary investigators.

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