

Input to the Lateral Habenula from the Basal Ganglia Is Excitatory, Aversive, and Suppressed by Serotonin

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SUMMARY

The lateral habenula (LHb) has recently been identified as a key regulator of the reward system by driving inhibition onto dopaminergic neurons. However, the nature and potential modulation of the major input to the LHb originating from the basal ganglia are poorly understood. Although the output of the basal ganglia is thought to be primarily inhibitory, here we show that transmission from the basal ganglia to the LHb is excitatory, glutamatergic, and suppressed by serotonin. Behaviorally, activation of this pathway is aversive, consistent with its role as an “antireward” signal. Our demonstration of an excitatory projection from the basal ganglia to the LHb explains how LHb-projecting basal ganglia neurons can have similar encoding properties as LHb neurons themselves. Our results also provide a link between antireward excitatory synapses and serotonin, a neuromodulator implicated in depression.

INTRODUCTION

Recent studies in monkeys have shown that neurons in the lateral habenula (LHb) become active when an animal fails to receive an expected reward or if the animal receives a signal indicating a negative outcome (Matsumoto and Hikosaka, 2007), i.e., these neurons encode “antireward” conditions and compute reward prediction errors—the difference between the amount of reward expected and the amount of reward received, a computation that is thought to drive reinforcement learning (Sutton and Barto, 1998). LHb neurons have also been shown to inhibit dopaminergic neurons in the ventral tegmental area (VTA) (Ji and Shepard, 2007), which encode “reward” conditions (Schultz, 1997; but see Matsumoto and Hikosaka, 2009). These findings are consistent with the view that an antireward (LHb) nucleus inhibits a reward (VTA) center and drives negative reward signals in dopamine neurons. However, the nature of the inputs that drive aversive responses in the LHb, as well as their possible modulation by other neurotransmitters, is poorly understood.

The globus pallidus internus (GPi), an output region of the basal ganglia, and its nonprimate homolog, the entopeduncular (EP) nucleus, are major sources of input to the primate (Kim et al., 1976; Parent et al., 2001) and rodent (Herkenham and Nauta, 1977) LHb, respectively, as well as the thalamus (Filion and Harnois, 1978; Harnois and Filion, 1982; Parent et al., 2001). The GPi and EP are thought to be primarily inhibitory brain regions because neurons in both structures express GABAergic molecular markers (Oertel et al., 1984; Stephenson et al., 2005), and lesions of the EP greatly reduce these markers in the LHb and thalamus (Penney and Young, 1981; Vincent et al., 1982). Recently, it was found that most LHb-projecting pallidal neurons have reward-modulated activity that begins before that of LHb neurons themselves, consistent with upstream control of LHb neurons (Hong and Hikosaka, 2008). Surprisingly, LHb-projecting pallidal neurons display antireward characteristics, similar to LHb neurons (Hong and Hikosaka, 2008). This finding suggests that either inhibitory projections out of the basal ganglia disinhibit LHb neurons or a previously unidentified excitatory projection exists from the basal ganglia to the LHb.

Here we test the hypothesis that an excitatory projection exists from the EP to the LHb that signals aversive events. We use a combination of optogenetics and immunohistochemistry to show that the projection from the EP to the LHb is predominantly excitatory, glutamatergic, and aversive. We also show that the excitatory projection from the EP to the LHb is suppressed by low concentrations of serotonin, providing a link between aversive signaling in the LHb and a neuromodulator involved in mood disorders.

RESULTS

Excitatory, Glutamatergic Projections from the EP to the LHb

To test the hypothesis that the projection from the EP to the LHb is excitatory, we injected AAV-driving expression of the light-inducible cation channel channelrhodopsin-2 (Boyden et al., 2005), tagged with yellow fluorescent protein (ChR2-YFP), into the EP in vivo. Two weeks after injection, we prepared coronal brain slices, which displayed localized fluorescence in neuronal cell bodies in the EP (Figure 1A and see Figure S1 available online) as well as fluorescent fibers in the projection region, the lateral aspect of the LHb (Figure 1B and Figure S1). To test for

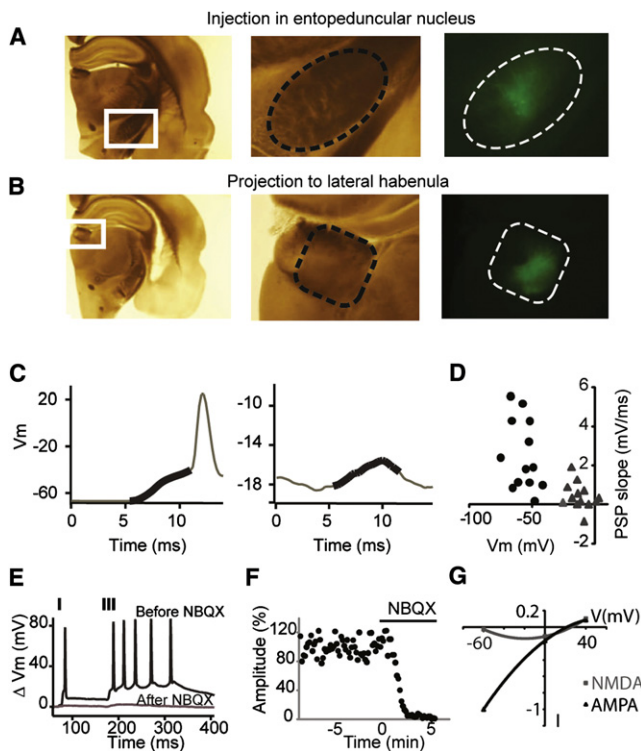


Figure 1. Stimulation of EP Axons in the LHb Excites LHb Neurons via Activation of Glutamate Receptors

(A) Example of injection of AAV-ChR2-YFP (green) into EP (insets). (B) Example of EP projections expressing ChR2-YFP in LHb (insets). Projections are from the same injection shown in (A) (see Figure S1 for higher-magnification pictures). (C) Left: light-evoked depolarization at resting potential. Right: light-evoked depolarization of same cell at depolarized potential. Light pulse (2 ms) delivered at $t = 0$. Bold line indicates regions used to compute the postsynaptic potential (PSP) slope. (D) Light-evoked PSP slopes at resting potential (circles) and depolarized potentials (triangles) for 13 cells. (E) Light-evoked responses reduced by NBQX (3 μ M). Light pulses indicated by marks above traces, here and in Figure 4. (F) Example of light-evoked excitatory currents at $V_{\text{clamp}} = -50$ mV blocked by NBQX (see Figure S1 for example of light-evoked GABA-A response). (G) I-V curves for NMDA (top, gray) and AMPA (bottom, black) responses fitted with second-order polynomials and normalized to the AMPA response at -60 mV ($n = 9$ cells).

excitation of LHb neurons by EP inputs, we obtained whole-cell current-clamp recordings from neurons in the lateral aspect of the LHb and stimulated the EP inputs to the LHb with brief (0.5–5 ms) pulses of 470 nm light through an LED-coupled optic fiber placed over the LHb. Consistent with the EP providing excitatory input to the LHb, light stimulation produced depolarizing synaptic responses at resting potentials (Figures 1C and 1D; 13/13 cells depolarized). To maximize detection of any hyperpolarizing synaptic response, we injected depolarizing current and raised the membrane potential close to 0 mV. Even in these conditions, the slope of the postsynaptic response remained positive for 12 out of 13 cells (Figures 1C and 1D), indicating dominant depolarizing synaptic input. Bath application of NBQX largely blocked the excitatory response, indicating its mediation by AMPA-type glutamate receptors (Figure 1E), although we could detect GABA-mediated currents when

cells were clamped at positive holding potentials (Figure S1). Voltage-clamp recordings in the presence of picrotoxin (100 μ M) showed that the excitatory EP input consisted of inwardly rectifying AMPA responses and minimal NMDA responses (Figures 1F and 1G).

To confirm the electrophysiological results, we injected in vivo the retrograde tracer cholera toxin subunit B conjugated with Alexa 488 (CTx⁴⁸⁸) into the LHb (Figure 2A), followed by immunohistochemistry of the EP. Consistent with the electrophysiological results, we found that about two-thirds of retrogradely labeled cell bodies in the EP expressed the vesicular glutamate transporter VGLUT2, a marker of glutamatergic neurons, and a minority expressed the GABAergic marker GAD67 (Figure 2B). These results indicate substantial excitatory, glutamatergic projections from the basal ganglia to the LHb, projections that probably contribute to the antireward responses of LHb neurons (Hong and Hikosaka, 2008; Matsumoto and Hikosaka, 2007).

Selective, Optical Stimulation of EP Inputs to the LHb Is Aversive

The majority of neurons in the basal ganglia that project to the primate LHb are excited by aversive stimuli, similar to LHb neurons themselves (Hong and Hikosaka, 2008). This suggests that output neurons of the basal ganglia that project to the LHb are driving LHb neurons' responses to aversive stimuli and predicts that stimulation of fibers from the EP to the LHb is aversive. To allow selective activation of the EP-LHb pathway in vivo, we injected AAV that drives expression of ChR2-YFP into the rat EP and implanted chronic dual fiberoptic cannulae that provided optical access to the LHb bilaterally (Figure S2). Three weeks later, we optically stimulated ChR2-YFP-expressing axons in the LHb (which originated from cell bodies in the EP) via a fiberoptic cable connected to the implanted cannulae and coupled to a blue laser. To determine whether stimulation of the EP-LHb pathway is aversive or rewarding, we tested rats for directed place preference by using a two-compartment (A and B) shuttle box (see Experimental Procedures and Figure 3A). During a baseline period of 10 min, the animals spent equal time in compartments A and B. Subsequently, during the next 30 min, light pulses (20 Hz) were delivered to the LHb when the animal was in compartment A. Animals developed a clear avoidance of compartment A during this period (Figure 3B). This aversive effect was reversible, because optogenetic activation of the EP-LHb pathway while animals were in compartment B reversed the avoidance (Figures 3C–3E); delivery of light alone had no effect (Figures 3F and 3G). These results indicate that the EP-LHb pathway provides aversive signals to the animal consistent with EP driving excitatory, antireward signals of the LHb.

Neuromodulation of Excitatory Input from the EP to the LHb

The LHb has been implicated in the pathophysiology of depression (Hikosaka, 2010; Li et al., 2011), potentially by reducing the output of brainstem aminergic neurons (Ferraro et al., 1996; Hikosaka, 2010; Ji and Shepard, 2007). However, the neuromodulation of transmission that drives LHb neurons is poorly understood. Because of its well-established role in depression, we tested whether serotonin could modulate the

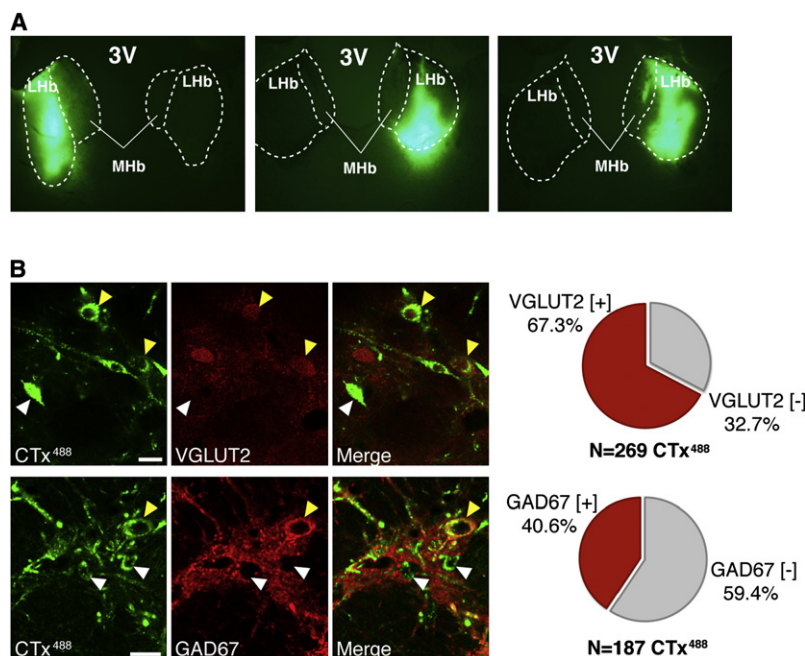


Figure 2. The Majority of LHb-Projecting EP Neurons Are Glutamatergic

(A) Cholera toxin (CTx⁴⁸⁸, green) injection sites. LHb, lateral habenula; MHb, medial habenula; 3V, third ventricle. (B) Confocal images of EP neurons retrogradely labeled by LHb injections of CTx⁴⁸⁸ (green) that contained (yellow arrowheads) or not (white arrowheads) for the glutamatergic marker, VGLUT2 (red, top), or the GABAergic marker, GAD67 (red, bottom). Scale bars represent 20 μ m.

Shepard, 2007), including dopaminergic neurons (Ji and Shepard, 2007). We show that the excitatory EP input to the LHb is suppressed by serotonin, suggesting that serotonin inhibits upstream synapses responsible for decreasing dopamine output. Our findings provide a link between a neuromodulator relevant to mood disorders and an antireward circuit.

Our discovery of a direct, glutamatergic projection from the EP to the LHb is consistent with a recent study showing expression of VGLUT2 mRNA in the EP (Barroso-Chinea et al., 2008). This study found high VGLUT2

mRNA expression in the rostral EP that preferentially targets the LHb (Araki et al., 1984) but also VGLUT2 mRNA in neurons that project to the thalamus. We extend this finding by demonstrating the presence of a strong, excitatory, glutamatergic projection from the EP to the LHb, as well as VGLUT2 expression in the majority of LHb-projecting EP neurons. We also show that stimulation of the excitatory projection from the EP to the LHb drive LHb neuronal responses to aversive events. Consistent with previous results (Oertel et al., 1984; Vincent et al., 1982), we also found electrophysiological and immunohistochemical evidence of a GABAergic projection from the EP to the LHb. Our immunohistochemistry suggested that a minority of LHb-projecting EP neurons are GABAergic, consistent with a previous pharmacohistochemical study (Araki et al., 1984), although we cannot rule out the possibility that our GAD67 antibody did not label all GABAergic EP neurons. The function of this GABAergic projection is unclear. It may control the gain of LHb neuronal activity or, given that LHb neurons are inhibited by unexpected, rewarding stimuli (Hong and Hikosaka, 2008; Matsumoto and Hikosaka, 2007), these inputs may signal the presence of reward. Future studies that selectively manipulate GABAergic inputs to the LHb are needed to address this issue.

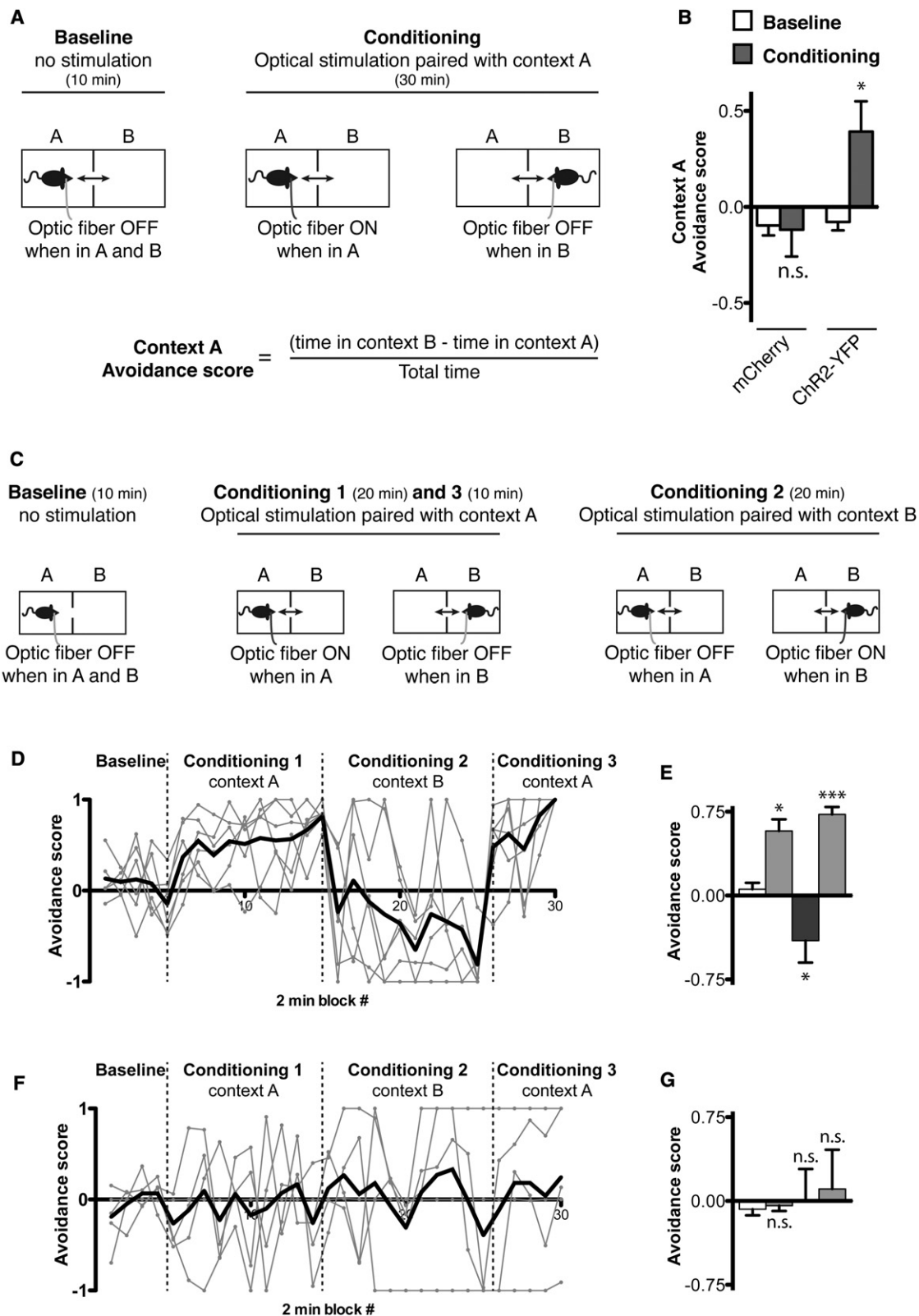
DISCUSSION

Here we investigate the physiological and behavioral function of basal ganglia outputs to the LHb by in vivo labeling of the EP nucleus with ChR2-YFP. We find that this pathway is primarily excitatory and glutamatergic and provides an aversive stimulus, consistent with upstream control of LHb antireward responses. Our results explain how a basal ganglia output, traditionally thought to be inhibitory (Oertel et al., 1984), can display similar encoding properties as its target nucleus, the LHb (Hong and Hikosaka, 2008).

We also examined the impact of serotonin on neurons in the LHb, a nucleus that provides inhibitory influence over brainstem aminergic nuclei (Ferraro et al., 1996; Hikosaka, 2010; Ji and

synaptic and/or intrinsic excitability of LHb neurons. We focused on neurons in the lateral aspect of the LHb, which received input from the ChR2-YFP-labeled EP. To examine the synaptic target of serotonin, we recorded responses to paired-light pulses (separated by 100 ms) in voltage clamp. Synaptic currents were reduced by bath application of low concentrations of serotonin (Figure 4A; $27\% \pm 6\%$ depression after first light pulse; $p = 0.001$; $n = 10$ cells), but not dopamine (Figure 4A; $7\% \pm 5\%$; $p = 0.26$; $n = 7$ cells), and the ratio of the second to first response increased after serotonin application (Figures 4B and 4C; $22\% \pm 9\%$ increase; $p = 0.02$), consistent with a reduction in the probability of neurotransmitter release. In contrast to serotonin's effect on synapses, we observed no change in the response to depolarizing current injection (Figures 4D and 4E; all $p > 0.1$; $n = 13$ cells) and no change in resting potential (V_m before serotonin, $-56\text{mV} \pm 2\text{mV}$; after serotonin, $-56\text{mV} \pm 2\text{mV}$; $p > 0.6$; $n = 13$ cells). These results indicate that serotonin provides presynaptic inhibition to excitatory input from the EP to the LHb.

We have isolated and characterized the synapses of a specific projection thought to participate in the computation of reward prediction errors—the difference between the amount of reward expected and the amount of reward received, a computation thought to drive reinforcement learning (Sutton and Barto, 1998). Given that both LHb-projecting basal ganglia neurons and LHb neurons are excited by the presence of unexpected, aversive events (Hong and Hikosaka, 2008; Matsumoto and Hikosaka, 2007) and our finding that projections from the basal ganglia to the LHb are excitatory and aversive, it is likely that



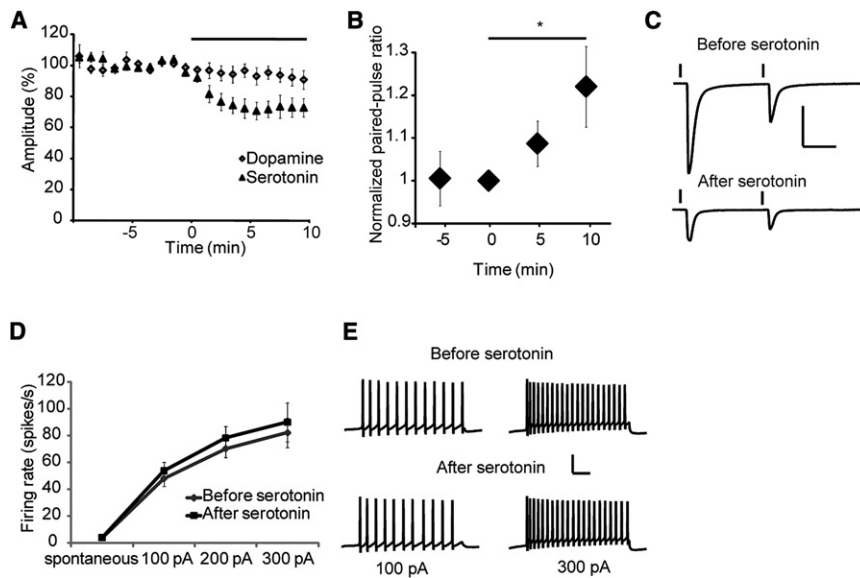


Figure 4. Serotonin Depresses Excitatory Input to LHB from EP

(A) Serotonin (1 μ M; $n = 10$ cells), but not dopamine (1–10 μ M; $n = 7$), depresses light-evoked excitatory currents. Bar indicates time of drug application. (B) Serotonin increases the paired-pulse ratio (i.e., amplitude of second response/amplitude of first response) of light-evoked excitatory currents ($n = 10$ cells). (C) Example of serotonin-mediated depression of light-evoked excitatory currents. Scale bar represents 100 ms, 200 pA. (D) No effect of serotonin (1 μ M) on spiking during current injection (as indicated; $n = 13$ cells). (E) Example of current-evoked spiking before and after serotonin. Scale bar represents 50 ms, 40 mV. * $p = 0.02$. All error bars represent SEM.

excitatory projections from the basal ganglia to the LHB signal negative reward prediction errors. Interestingly, the activity of these excitatory projections was suppressed by low concentrations of serotonin. This suggests that synapses that transmit reward prediction errors are subject to neuromodulation and opens new avenues for the study of the interaction between mood and learning.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats, age 28–32 days at time of surgery (42–46 days when sacrificed for slice experiments), were housed one to four/cage and kept on a 12/12 hr light-dark cycle (lights on/off at 6 a.m./6 p.m.). 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 the flexed version of ChR2(H134R)-eYFP was kindly provided by Dr. Karl Deisseroth (Stanford). To make the nonflexed version, we PCR amplified ChR2(H134R)-eYFP sequence flanked by KpnI and EcoRI sites and subcloned them into pAAV-EF1 α -flexed-ChR2(H134R)-eYFP-WPRE vector by 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.

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 to -6.9 mm from dura). We injected 0.1 – 0.5 μ l of virus into each hemisphere over 8–20 min by using a picospritzer. Control rats for behavior experiments were injected with an AAV encoding mCherry. The injection pipette was not removed until 10 min 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) were implanted in order to have the tip of the fiberoptic cannulae (200 μ m, 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) (see Figure S2) and were 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

Rats ($n = 7$ in ChR2-YFP group, $n = 5$ in mCherry control group) used for directed place preference (DPP) underwent surgery at 4–7 weeks old, and behavior experiments were conducted at least 3 weeks after surgery. DPP was carried out in a shuttle box (50 cm wide $\times$ 25 cm deep $\times$ 30 cm high; 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 by using an optical fiber coupled to a 473 nm solid-state laser diode (OEM Laser Systems) with 20 mW of output from the 200 μ m 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

Figure 3. Stimulation of EP Axons in the LHB Is Aversive

(A) Schematic of the directed place preference paradigm (see Experimental Procedures for details). Rats were allowed to move between both sides of the test cage during the entire 40 min session. No stimulation was delivered during the 10 min baseline period. After this baseline period, optical stimulation of EP axons in the LHB was delivered when the rat was in context A (see Figure S2 for surgical design and location of optic fiber cannulae). (B) Light has no effect in mCherry control group but causes avoidance of context A in rats expressing ChR2-YFP (ChR2-YFP, $n = 7$; mCherry, $n = 5$). (C) Schematic of the reversal of directed place preference (see Experimental Procedures for details). Throughout the entire 1 hr session, the rat was allowed to move freely between both sides of the test cage. No stimulation was delivered during the first 10 min baseline period. After this baseline period, optical stimulation of EP axons in the LHB was delivered every time the rat was in context A. This optical stimulation paired with context A lasted 20 min. After this period, optical stimulation was reversed and now paired with context B for the next 20 min and then paired back again with context A for the last 10 min. (D) Individual avoidance scores (gray lines) over time and mean (dark line) for ChR2-YFP-expressing rats. (E) Mean avoidance scores for ChR2-YFP-expressing rats for each period ($n = 6$ rats). (F) Individual avoidance scores for mCherry-expressing rats as in (D). (G) Mean avoidance scores for mCherry-expressing rats for each period ($n = 5$ rats). n.s., not significant; * $p < 0.05$, *** $p < 0.001$. All error bars represent SEM.

preference was found during this first 10 min. After this 10 min baseline period, optical stimulation (continuous 20 Hz, 5 ms 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 s). 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 another 20 min and then paired again with context A for the last 10 min of the 1 hr session (see schematic in Figure 3). One ChR2-YFP-expressing rat lost its cannula before the DPP reversal test, so only six 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 min to baseline period (0–10 min).

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, and 3.1 mM pyruvic acid; gassed with 95% O₂/5% CO₂) and cut in 400- μ m-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, and 4 mM CaCl₂ [pH 7.4]; 22°C–25°C; gassed with 95% O₂/5% CO₂) for 30 min. After an additional 30 min of recovery at room temperature (RT), 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, by using an Axopatch 1D amplifier with a 5 kHz sampling frequency and a filter set at a –3 dB cutoff frequency of 5 kHz. For current-clamp recordings, the intracellular solution consisted of 130 mM K-Gluconate, 5 mM KCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₃GTP, 10 mM Na-phosphocreatine, and 0.6 mM EGTA (pH 7.2). For voltage-clamp recordings, the intracellular solution consisted of 7.5 mM QX314, 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl₂, 4 mM Na₂-ATP, 0.4 mM Na-GTP, 10 mM Na-phosphocreatine, and 0.6 mM EGTA (pH 7.2). We delivered 470 nm light pulses to the LHB via an optic fiber attached to an LED. To examine the effect of serotonin (1 μ M in all experiments) on excitability, we also injected current (–100, 0, 100, 200, and 300 pA) for 300 ms—one current injection every 10 s in current clamp. To examine the effect of serotonin on light-evoked currents, we clamped cells at –50 mV, and 0.5–5 ms light pulses were given in pairs, 100 ms between each pulse, every 10 s. Picrotoxin (100 μ M) was included in the extracellular recording solution during voltage-clamp but not current-clamp recordings. For I–V recordings, NBQX (3 μ M) and APV (100 μ M) were added during the recordings taken at –60 mV, 0 mV, and 40 mV (minimum ten 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

For measurement of the effects of EP fiber stimulation on membrane potential for each cell, the slope of the postsynaptic potential (PSP) was measured (average of three sweeps) at resting potential. The same start and peak times were used to compute the PSP slope during depolarization (average of three 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.

Tracer Injection, Perfusion, and Tissue Processing

To label LHB-projecting EP neurons, we unilaterally injected 0.5 μ l of cholera toxin subunit B conjugate to the Alexa Fluor 488 (CTx⁴⁸⁸) (2 mg/ml in phosphate-buffered saline, PBS [pH 7.4]) in the LHB (AP: –3.6 mm, ML: 0.7 mm, DV: –4.8 mm) over 5–7 min. Rats were allowed to survive for 40 hr, were perfused, and their brains were processed for immunohistochemistry.

For perfusion, rats were deeply anesthetized by using a mix of ketamine/dexdomitor (75 and 5 mg/kg, respectively intraperitoneally) and transcardially perfused with saline followed by a solution of 0.1 M phosphate buffer (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 3 days. Frozen brains were sectioned at 50 μ m with a sliding microtome in the coronal plane.

For each brain, three 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 hr. 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 hr at RT. After three washes in TN buffer, slices were incubated with secondary antibody Alexa Fluor 647 goat anti-mouse (Invitrogen) in TN/3% NGS/0.2% Triton X-100 for 4 hr at RT. Slices were washed and mounted by using Vectashield mounting medium (Vector Laboratories). Images were taken with a FV1000 confocal microscope (Olympus), adjusted for brightness by using Fluoview software, and assembled in Adobe Illustrator.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.neuron.2012.02.037.

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