Lateral Habenula Involvement in Impulsive Cocaine Seeking

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The lateral habenula (LHb) is a brain structure receiving inputs from limbic forebrain areas and innervating major midbrain monoaminergic nuclei. Evidence indicates LHb involvement in sleep control, reward-based decision making, avoidance of punishment, and responses to stress. Additional work has established that the LHb mediates negative feedback in response to aversive events. As a hallmark of drug addiction is the inability to limit drug use despite negative consequences, we hypothesize that LHb dysfunction may have a role in the lack of control over drug seeking. Here we examine the effects of LHb inactivation in control over drug seeking in several cocaine self-administration (SA) paradigms in rats. We find that inhibition of the LHb with GABAergic agonists did not alter cocaine SA under progressive ratio or seeking/taking chained reinforcement schedules, or during punishment-induced suppression of cocaine-reinforced responding. In contrast, LHb inhibition increased cocaine seeking when the drug was not available in rats trained to discriminate its presence using an environmental cue. This effect of LHb inhibition was selective for cocaine, as it did not impair responding for sucrose reinforcement. The effect of LHb injection of GABA agonists was mimicked by intra-LHb muscarinic cholinergic (mACh) antagonist injection, and activation of mACh receptors excited a majority of LHb neurons in *in vitro* electrophysiology experiments. These results indicate that the LHb participates in the suppression of impulsive responding for cocaine through the activation of a cholinergic circuit, and they suggest that LHb dysfunction may contribute to impaired impulse control associated with drug addiction.
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INTRODUCTION

The habenular complex constitutes a major relay between the forebrain and midbrain/hindbrain areas (Lecourtier and Kelly, 2007; Sutherland, 1982). Two major subdivisions have been identified based on anatomical connectivity and cellular composition: the lateral and the medial habenula (LHb and MHb, respectively). The LHb receives input from the limbic forebrain and basal ganglia and influences the activity of all major mid-brain monoaminergic nuclei. As LHb neurons are activated by negative outcomes, such as punishment, omission of an expected reward, or stimuli associated with negative events, it is suggested that a primary role is to process negative feedback associated with reward seeking behavior (Matsumoto and Hikosaka, 2007, 2009). Consistent with this, activation of the LHb strongly inhibits dopamine (DA) neurons of the ventral tegmental area (VTA), a central component of the brain's reward pathway (Christoph *et al*, 1986; Ji and Shepard, 2007; Matsumoto and Hikosaka, 2007). However, this inhibition of DA neurons is indirect, involving LHb-mediated excitation of inhibitory neurons in the rostromedial tegmental area (RMTg), that broadly innervate

the VTA DA neurons (Hong *et al*, 2011; Jhou *et al*, 2009). As optogenetic activation of the LHb to RMTg pathway is aversive (Stamatakis and Stuber, 2012), it is proposed that regulation of VTA DA activity by the LHb may underlie aversive effects of abused drugs (Lecca *et al*, 2014; Velasquez *et al*, 2014). Consistent with this, LHb neurons are influenced by cocaine (Good *et al*, 2013; Jhou *et al*, 2013; Maroteaux and Mameli, 2012; Zuo *et al*, 2013), and LHb activation is linked to the delayed aversive effects of cocaine (Jhou *et al*, 2013), as well as to the negative, depressive-like state that occurs after chronic cocaine (Meye *et al*, 2015). However, evidence for involvement of the LHb in ongoing drug-seeking behavior is limited (Lecca *et al*, 2014; Velasquez *et al*, 2014). As the LHb is associated with the aversive properties of abused drugs (Jhou *et al*, 2013) and the suppression of behavior leading to negative outcomes (Hikosaka, 2010), we hypothesize that it may be involved in regulating excessive or inappropriate drug-seeking behavior seen in addiction, where drug seeking persists despite its adverse consequences.

Previous studies have used animal behavioral paradigms to model diagnostic criteria of addiction in humans (Belin-Rauscent *et al*, 2016; Vanderschuren and Ahmed, 2013). We have adapted rat cocaine SA paradigms (Deroche-Gamonet *et al*, 2004; Pelloux *et al*, 2007; Vanderschuren and Everitt, 2004) to examine whether LHb inhibition alters: (i) motivation to seek cocaine measured by break points in a progressive ratio (PR) schedule; (ii) the persistence of cocaine seeking during punishment of cocaine-reinforced responding; or (iii) the ability to control cocaine seeking

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during alternating periods of signaled availability and non-availability (Go/NoGo task). We find that LHb inhibition increases cocaine seeking when cocaine is not available in a Go/NoGo paradigm, suggesting impaired control over drug seeking.

MATERIALS AND METHODS

Subjects

Male Long Evans rats (Charles River Laboratories, Wilmington, MA) weighing 300–400 g at the beginning of experiments were housed 2–3 per cage in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. They were maintained in a temperature- and humidity-controlled environment, under a reverse 12 h light/dark cycle, with *ad libitum* food and water. All behavioral experiments were conducted during the dark cycle. The experimental procedures were approved by the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health (Rockville, MD) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals provided by the NIH and adopted by the NIDA Intramural Research Program.

Surgery

Rats were anesthetized with Equithesin (1% pentobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propyleneglycol, 11% ethanol, 3 ml/kg i.p.), and a silastic catheter (ID: 0.020” OD: 0.037”, Dow Corning, MI) was advanced 3.5 cm into the right jugular vein. The catheter terminated in an L-shaped steel tube mounted on top of the skull with cranioplastic cement and was secured with three stainless steel screws. In the same surgery, subjects were stereotaxically implanted with bilateral guide cannulae (C315, Plastics One, Roanoke, VA) aimed 1 mm dorsal to the LHb (AP: − 3.6, L: ± 0.6, V: − 3.6 mm relative to bregma). Following surgery, rats were housed individually and allowed to recover for at least 1 week prior to beginning experiments. Catheters were flushed after every training session with 0.1 ml of heparinized saline and 120 mg/ml cefazolin throughout the study.

Behavioral Procedures

Operant training took place in standard rat operant chambers (Med-Associates; St Albans, VT) placed inside sound attenuated boxes and equipped with a wall-mounted ventilation fan that provided background noise. A liquid swivel was mounted above the operant box to allow for connecting the IV catheters via PE50 tubing to an infusion pump located outside the chamber. The operant boxes were equipped with liquid dippers able to dispense 10 μl of a liquid solution to a magazine situated between two retractable levers. A light was located above each lever and a house light was placed on the opposite wall.

Experiment 1. Rats were trained on a drug-seeking/taking chained schedule of cocaine self-administration (Zapata *et al*, 2010) in which they were required to press one lever (taking lever) to receive an i.v. cocaine infusion (0.75 mg/kg/

infusion) under a fixed ratio 1 (FR1) schedule (Zapata *et al*, 2010). Following each response, the taking lever was retracted and the house light extinguished, followed by illumination of a cue light above the taking lever for 30 s. Animals were allowed to self-administer for a maximum of 40 infusions or 2 h. A chained schedule was then initiated in which every infusion cycle started with the insertion of a second lever, designated as the drug-seeking lever. The first lever press on the seeking lever after completion of a random interval (2 s average, RI2) resulted in retraction of the seeking lever and extension of the taking lever. The next press of the taking lever resulted in the cocaine infusion sequence. A time out of 30 s (TO30) was imposed after every infusion, after which another infusion cycle started with the presentation of the drug-seeking lever. Thus rats self-administered cocaine under an RI2/FR1:TO30 chained cocaine seeking/taking schedule. Each daily session lasted for 2 h or 10 infusions. The RI and TO components of the chained schedule were then increased in the following steps; RI2/FR1:TO30, RI20/FR1:TO120, RI60/FR1:TO300, RI120/FR1:TO600. After stable responding on the final chained schedule was achieved, motivation for the drug was tested in two drug-seeking/taking sessions alternating with two sessions in which only the taking lever was present and cocaine was available under a PR schedule.

Intracranial infusion of drugs was carried out using a microinjection cannula (C315I, Plastics One) inserted into the surgically implanted guide cannula and extended 1.0 mm below its tip. One of the solutions (0.5 μl) was then infused at a rate of 1 μl/min. The microinjection cannula was removed 1 min after the infusion, and animals were then placed in the operant boxes. In Experiment 1, rats received either phosphate-buffered saline (PBS) or Baclofen/Muscimol (B/M, 1 mM/0.1 mM) infused bilaterally in the LHb immediately before each test session, and each animal was tested with both PBS and B/M in each test condition, in a random order, according to a within-subject repeated-measures design.

During PR schedule sessions, only the drug-taking lever was present and each infusion resulted in an increase in the response ratio requirement for the next infusion (Richardson and Roberts, 1996). The PR session was terminated when the animal failed to earn an infusion for 60 min. The last ratio completed was considered the breakpoint and was recorded as a measure of the animal's motivation to seek the drug. During the seeking/taking chained schedule sessions, the seeking responses were recorded as an additional measure of motivation for the drug thought to be less influenced by the non-specific motor enhancing effects of psychostimulants (Olmstead *et al*, 2000).

After testing for drug motivation, rats were given three sessions on the RI120/FR1:TO600 chained schedule, followed by five punished sessions in which a response on the seeking lever resulted in either access to the taking lever or a scrambled foot shock delivered through the floor grid (50% probability, 0.6 mA × 0.5 s). One group of animals received PBS and the other B/M infusions in the LHb before each session in a between-subject design.

Experiment 2. Rats self-administered cocaine for 12 sessions (2 h or 40 infusions per session) on an FR1

schedule. Then they were trained on a Go–NoGo task consisting of 2 h sessions comprised of 6 20 min alternating intervals of cocaine availability (Go)/non-availability (NoGo), signaled by the house light (light on during cocaine availability). During Go intervals, responses were reinforced with cocaine under an FR5 schedule, and during NoGo intervals, lever responses did not trigger cocaine infusions. The number of lever responses during the 3 Go and 3 NoGo intervals in each session was recorded. Training progressed until stable discrimination of the Go–NoGo intervals was observed (3 consecutive sessions in which NoGo responses were < 30% of total, 12–14 sessions). After reaching criterion, each rat received bilateral infusions of PBS or B/M in the LHb, in a randomized order, immediately before each test session. Sessions were counterbalanced such that some started with a Go period, while other began with a NoGo interval to control for the motor stimulant effects of cocaine.

Following testing on the Go–NoGo task, rats were retrained on an FR1 schedule and tested for sensitivity to punished responding for cocaine self-administration. They were divided into two groups, one group receiving bilateral LHb infusions of PBS and the other B/M prior to each session. Rats completed 3 baseline sessions, followed by 3 punished sessions in which cocaine infusions were accompanied by a scrambled foot shock 30% of the time ($0.6 \, mA \times 1 \, s$).

Experiment 3. Groups of rats were trained to self-administer cocaine in the Go–NoGo task. Then the effects of bilateral LHb infusion of one of the several neurotransmitter receptor antagonists was investigated. Each group received vehicle, B/M, or one of the two additional drugs on different test days in random order. These included the non-selective DA receptor antagonist flupenthixol (60 mM), the non-selective ionotropic glutamate receptor antagonist kynurenic acid (KA; 50 mM), a mixture of the 5-HT receptor antagonists ritanserin/ondansetron (5 mM/5 mM in 10% DMSO), the β-subunit-containing nicotinic acetylcholine (ACh) receptor antagonist mecamylamine (100 mM), or the non-selective muscarinic ACh (mACh) antagonist scopolamine (50 mM). The concentrations of all antagonists used in these experiments were chosen based on previous literature (de Mello Cruz et al, 2005; Murray et al, 2012; Santini et al, 2012; Sun et al, 2005; Tuerke et al, 2012; Wickham et al, 2013).

Experiment 4

In vitro *patch clamp electrophysiology.* Brain slices were prepared from 10-week-old rats using a vibrating tissue slicer (Leica VT1000S) according to previously published protocols (Jhou et al, 2013). Coronal slices (280 μm) containing the LHb were transferred to a holding chamber containing normal aCSF consisting of (mM): NaCl,126; KCl, 3.0; MgCl$_2$, 1.5; CaCl$_2$, 2.4; NaH$_2$PO$_4$, 1.2; glucose, 11.0; NaHCO$_3$, 26, saturated with 95% O$_2$/5% CO$_2$, at 35 °C for 20–25 min, maintained at room temperature. A brain slice was submerged in a low-volume (170 μl) recording chamber integrated into a fixed stage of an upright microscope and continuously perfused with warm (30–32 °C) aCSF at 2 ml/min using a peristaltic pump. The aCSF was warmed using

an inline solution heater (TC-324B, Warner Instruments). Drugs were prepared as stock solutions in H$_2$O and diluted in aCSF to the indicated concentrations. Visualization of LHb neurons was performed using differential interference contrast microscopy (Zeiss Axioskop). Recording electrodes (3–5 MΩ) were filled with (in mM): K-gluconate, 140; KCl, 5; HEPES, 10; EGTA, 0.2; MgCl$_2$, 2; Mg-ATP, 4; Na$_2$-GTP, 0.3; Na$_2$-phosphocreatine, 10; pH 7.2 with KOH. Whole-cell voltage clamp recordings were performed using an Axopatch 200B (Molecular Devices), WinLTP software (WinLTP Ltd), and an A/D board (National Instruments PCI-6251) residing in a personal computer. Series resistance was monitored throughout recordings using brief hyperpolarizing steps (−10 mV, 200 ms), and cells demonstrating > 10% change were excluded from analyses. Unless otherwise indicated, cells were voltage clamped at − 60 mV.

Drug application to LHb slices. Oxotremorine-M (Oxo-M) and scopolamine were purchased from Tocris Bioscience (Bristol, UK), dissolved at their final concentrations in normal aCSF and delivered to the brain slices via the peristaltic pump used for bath perfusion.

Experiment 5. A group of drug-naive rats was trained to respond for sucrose solution reward (10 μl of 20% sucrose) obtained via a dipper cup located in the food magazine. Lever presses on the active lever resulted in refilling of the dipper cup, followed by retraction of both levers and illumination of a cue light above the active lever for 5 s. Animals were trained under FR1 for a single 6-h session and then switched to 2 h long sessions or until 600 reinforcers were obtained. After 10 sessions, animals were switched to a Go–NoGo schedule (3 × 20 min intervals of food access interspersed with 3 × 20 min of sucrose unavailability, signaled by house light on or off, respectively). After stable responding was achieved (10–12 sessions), animals received bilateral infusions of either PBS or B/M in the LHb immediately before each daily session. Treatments were given over consecutive testing sessions in a random order in a within-subject design.

Histology

At the end of the experiment, cresyl violet (1% × 0.5 μl) was bilaterally infused through the guide cannulae. The animals were then killed, and their brains were removed and frozen on dry ice. Histological verification of the microinjection site was carried out in 30 μm cryostat frozen sections.

Data Analysis

Statistical analysis was conducted using one- or two-way repeated-measures ANOVA (GraphPad Software, La Jolla, CA). Main factors were training session/condition and treatment (LHb intracranial infusion). Differences among groups were assessed by Holms–Sidak's multiple comparison tests. An alpha value of $p < 0.05$ was considered statistically significant.
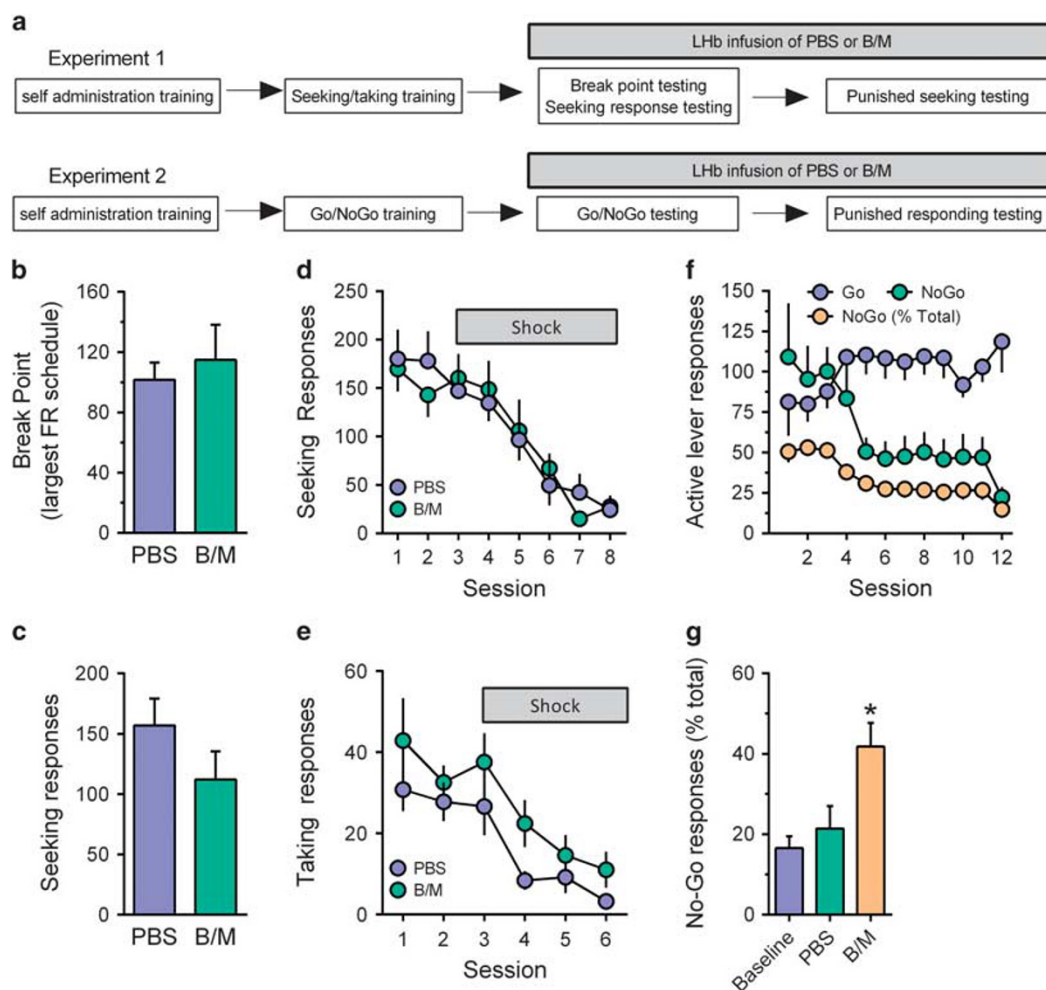
Figure 1 (a) Design of cocaine self-administration experiment 1 and experiment 2 (See Methods for more detail). (b) Effects of LHb transient inhibition with B/M on the break point in a progressive ratio schedule, and (c) the number of seeking responses in seeking/taking chained schedule in rats responding for IV cocaine infusions ($n=9$). (d) Effects of LHb transient inhibition with B/M on punished responding (electric foot shock) in a seeking/taking chained schedule ($n=5$ per group) or (e) in an FR1 taking schedule ($n=5$, PBS; $n=7$, B/M). (f) Acquisition of the discrimination between reinforced and non-reinforced components in a Go–NoGo task in rats self-administering cocaine ($n=12$). (g) Effects of LHb transient inhibition with B/M on responding on the active lever during NoGo intervals (*$p<0.05$ vs PBS control, paired t-test, $n=12$). All data are mean±SEM.

RESULTS

Histological examination of the injection sites showed that most infusions were made in the LHb, mainly in its medial aspect (Supplementary Figure S1). Only animals with a confirmed LHb infusion site were included in the data analysis.

Experiment 1: Inactivation of the LHb Does not Alter Motivation for Cocaine or Punished Responding for Cocaine

The sequence for experiment 1 is shown in Figure 1a. The effect of LHb inhibition with B/M was examined on cocaine-seeking behavior using a PR schedule of cocaine reinforcement. Bilateral B/M infusions did not significantly alter the maximum number of responses emitted to receive cocaine reinforcement (break point) ($t_{(8)}=0.51$, $p=0.62$, paired t-test, Figure 1b) nor the number of responses in the seeking

link of a seeking/taking chained schedule ($t_{(8)}=1.4$, $p=0.20$, paired t-test, Figure 1c).

We next evaluated whether LHb inhibition altered punished suppression of responding for cocaine by pairing seeking responses with foot shock in 50% of these trials. The punishment caused a significant decrease in subsequent seeking responses in both groups (main effect of punishment, $F_{(7,56)}=17.70$, $p<0.0001$, ANOVA, Figure 1d). However, inhibition of the LHb by B/M did not alter this effect (no main B/M treatment effect nor punishment by treatment interaction, $F_{(1,8)}=0$, $p=0.95$ and $F_{(7,56)}=0.48$, $p=0.84$, respectively). In an alternative punishment paradigm in which cocaine-taking responses were paired with foot shock, there was also decreased responding ($F_{(5,50)}=10.0$, $p<0.0001$, ANOVA, Figure 1e). However, the suppression of taking responses by punishment was also not altered by LHb inhibition (no main B/M treatment effect nor punishment by treatment interaction, $F_{(1,10)}=2.80$, $p=0.12$ and $F_{(5,50)}=0.26$, $p=0.93$, respectively). These data suggest that LHb inactivation does not alter the motivation to seek

cocaine nor the suppression of cocaine seeking by punishment.

Experiment 2. Persistence of Cocaine Seeking Despite its Signaled Unavailability After LHb Inhibition

The inability to refrain from drug seeking is a hallmark of addiction that can be modeled in rodents by testing their ability to withhold responding during signaled periods when the drug is not available (Belin et al, 2008; Deroche-Gamonet et al, 2004). We examined a role for the LHb in withholding responses for cocaine using a Go–NoGo task. During training, the percentage of incorrect responses emitted when cocaine was not available (NoGo) declined significantly from 50.4% on day 1 to 14.8% on day 12 ($F_{(11,121)} = 6.740$, $p = 0.0001$, one way ANOVA, Figure 1f). This resulted from decreased responding during NoGo periods ($F_{(11,121)} = 3.07$, $p = 0.04$, one way ANOVA, Figure 1f), with no change in responding during the Go intervals ($F_{(11,121)} = 1.68$, $p = 0.16$). LHb inactivation with B/M disrupted this learned discrimination, resulting in significantly increased cocaine-seeking responses during the NoGo period (PBS = 21.4, 9.1–33.8; B/M = 41.8, 29.0–54.7, mean and 95% confidence intervals; $t_{(11)} = 3.061$, $p = 0.01$, paired t-test, Figure 1g). Detailed analysis revealed that B/M infusions enhanced responding during NoGo intervals, without significantly altering responding during Go intervals (significant main effect of Go–NoGo intervals, $F_{(1,11)} = 16.74$, $p < 0.002$; and intervals × LHb infusion interaction, $F_{(1,11)} = 5.09$, $p = 0.045$, two way ANOVA, Figure 2a). We also observed a non-significant

(main effect of LHb infusion, $F_{(1,10)} = 2.52$, $p = 0.14$, two way ANOVA) trend toward increased responding on the inactive lever after B/M infusions (Figure 2b) during both Go and NoGo intervals (non-significant LHb infusion × intervals interaction, $F_{(1,10)} = 0.39$, $p = 0.55$, two-way ANOVA).

We next determined whether the effect of B/M on NoGo responding was present across all 3, 20 min periods in the test session. A three-way ANOVA with LHb infusion, Go–NoGo condition, and interval order as main factors revealed a significant Go–NoGo × LHb infusion interaction ($F_{(1,132)} = 5.08$, $p = 0.03$), with no significant effect of interval order ($F_{(2,132)} = 0.88$, $p = 0.42$) nor interval order × Go–NoGo × LHb infusion ($F_{(2,132)} = 0.11$, $p = 0.89$), indicating that the enhanced cocaine-seeking responses during the NoGo intervals was present throughout the entire test session (Figure 2c).

In the design above, all NoGo intervals were preceded by Go intervals in which cocaine was self-administered, perhaps leading to a confound in which the continued responding during NoGo periods resulted from cocaine-associated motor stimulation. To test this, the order of the Go–NoGo intervals was reversed, and sessions begun with a NoGo interval in which responding was measured before cocaine exposure. However, even with reversal of Go–NoGo order, responding for cocaine was still significantly elevated during NoGo trials (Figure 2d, significant Go–NoGo × LHb infusion interaction, $F_{(1,132)} = 14.5$, $p < 0.001$, three-way ANOVA). Importantly, the interval order did not influence this effect (non-significant interval order effect, $F_{(2,132)} = 0.16$, $p = 0.85$ nor interval order × Go–NoGo × LHb infusion interaction ($F_{(2,132)} = 0.31$, $p = 0.73$, Figure 2d), indicating that exposure to cocaine during the Go intervals did not influence responding during NoGo intervals.

To control for diffusion of B/M into brain areas dorsal to the LHb, we implanted cannulae with tips located 1 mm above the LHb and tested the effects of either the GABA agonists or PBS on the GoNoGo task ($n = 4$). Infusions of B/M at this anatomical site did not significantly modify NoGo responding ($t_{(3)} = 0.58$, $p = 0.60$, paired t-test, Supplementary Figure S2) compared with PBS, suggesting that diffusion of B/M up the infusion track to areas dorsal to the LHb did not account for the pattern of results observed with intra-LHb infusions.

Experiment 3. Go–NoGo Deficits are Mimicked by mACh Receptor Antagonism

We next investigated the neurotransmitter pathways that may underlie LHb control of cocaine seeking during NoGo periods. Local LHb blockade of ionotropic excitatory amino-acid receptors with KA (50 mM), or D1 and D2 DA receptors with flupenthixol (60 mM), did not alter cocaine seeking in the Go–NoGo procedure (Figure 3a). Similarly, blockade of 5-HT$_2$ or 5-HT$_3$ receptors with ritanserin and ondansetron (5 mM each), respectively, did not alter responding (Figure 3b). To investigate the involvement of cholinergic receptors, we tested the β-subunit nACh receptor antagonist mecamylamine (100 mM), which did not alter responding during NoGo intervals. In contrast to these negative results, intra-LHb infusion of the mACh receptor antagonist scopolamine (50 mM) significantly increased
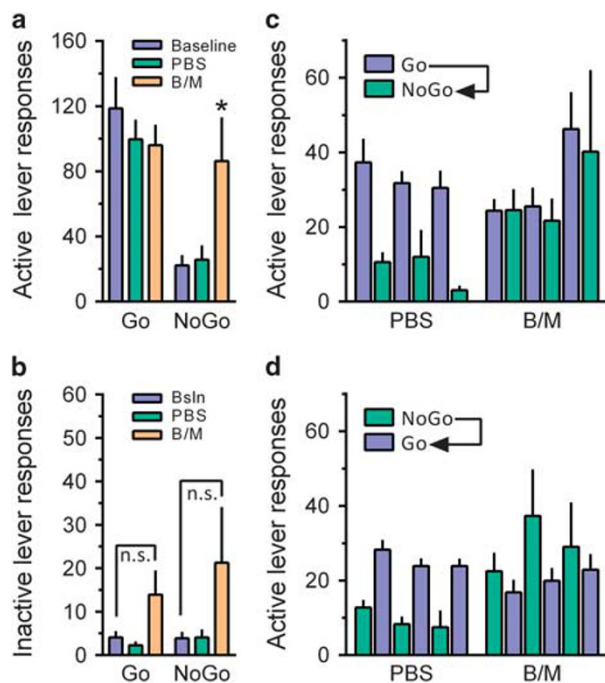
Figure 2 Effects of LHb transient inactivation on the total number of responses on the active (a) or inactive (b) lever during the Go and No-Go intervals (*$p < 0.05$ vs PBS control, Holm–Sidak's test, $n = 12$). (c) Active lever responses during each consecutive 20 min alternating Go–NoGo periods, starting the session with a Go or NoGo (d) interval, after PBS or B/M infusion in the LHb ($n = 12$ in both experiments). All data are mean ± SEM.
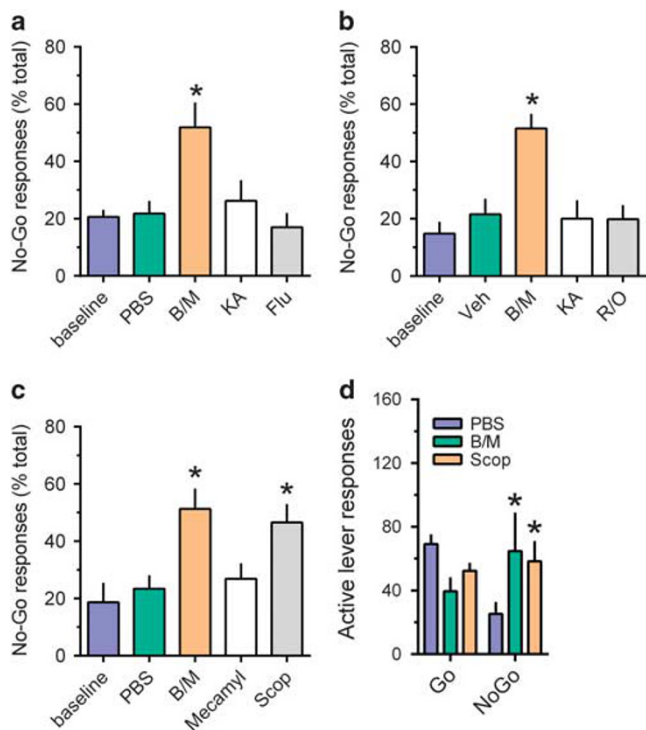
Figure 3 Effects of LHb infusion of receptor antagonists on responding during the NoGo intervals. (a) Effects of the ionotropic glutamate receptor antagonist kynurenic acid (KA, 50 mM) and the dopamine antagonist flupenthixol (Flu, 60 mM), compared with the effects of PBS and B/M (1 mM/0.1 mM). $n = 8$ for all groups. (b) Effects of KA (50 mM) and the combination $5HT_{2/3}$ antagonist ritanserin/ondansetron (R/O, 5 mM each), $n = 7$ for all groups. (c) Effects of the nicotinic antagonist mecamylamine (Mecamyl, 100 mM) and the muscarinic antagonist scopolamine (Scop, 50 mM), $n = 11$ for all groups. (d) Effects of LHb scopolamine infusions on the total number of responses during the NoGo and Go intervals ($n = 11$). All data are mean ± SEM (*$p < 0.05$ vs vehicle group, Holm–Sidak's test).

cocaine-seeking responses during NoGo intervals ($F_{(2,25)} = 6.21$, $p = 0.004$, ANOVA, $p = 0.02$ vs PBS, Holms–Sidak's *post hoc* test, Figure 3c). Further analysis revealed that scopolamine selectively increased responding during NoGo intervals without affecting responses during Go intervals (significant LHb infusion × Go–NoGo interaction, $F_{(2,20)} = 5.794$, $p = 0.01$, two way ANOVA, Figure 3d).

Experiment 4. mACh Receptor-Induced Excitation of LHb Neurons *In Vitro*

Our observation that B/M infusions into the LHb disrupt Go–NoGo responding for cocaine imply that increased activity of these neurons is required for this discrimination. This, together with the results with scopolamine, led us to hypothesize that mACh receptor activation excites LHb neurons. To test this, whole-cell patch clamp recordings were obtained from LHb neurons in brain slices and the effects of the mACh agonist Oxo-M on the amount of current necessary to hold the neuronal membrane potential (I_{hold}) at −60 mV was examined. Most LHB neurons (50%) exhibited a decrease in I_{hold} upon application of Oxo-M (10 μM; Figure 4), which is consistent with the activation of monophasic depolarizing inward currents. The remaining
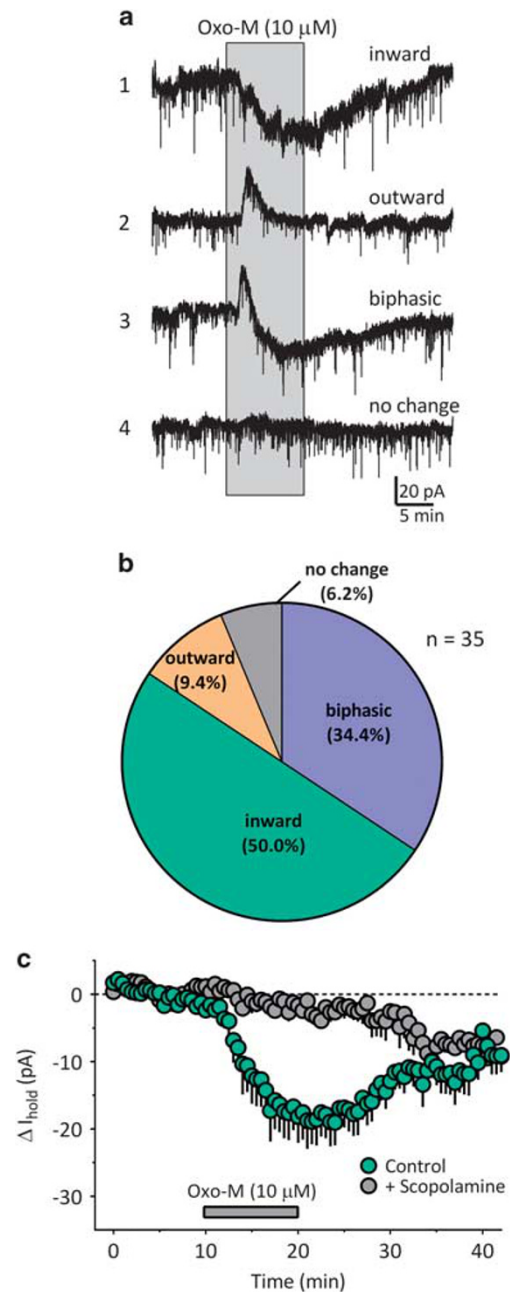
Figure 4 Muscarinic acetylcholine (mACh) receptor control of LHb neurons. (a) Representative examples of four distinct membrane current responses that were observed upon application of mACh receptor agonist (oxotremorine-M (Oxo-M) 10 μM) in four different LHb neurons. The recordings were made in brain slices with neuronal membrane potentials voltage clamped at −60 mV. After stable baseline membrane current was observed for at least 10 min, Oxo-M was applied for 10 min (gray vertical bar) and membrane current was recorded for the next 20 min. (b) Proportion of LHb neurons displaying each of four different responses to bath-applied Oxo-M in *in vitro* voltage clamp experiments. These responses to Oxo-M included excitatory inward currents, inhibitory outward currents, biphasic outward-inward currents, and no change in membrane current. Note that inward depolarizing currents were most frequently observed upon Oxo-M application. (c) The non-selective mACh receptor antagonist scopolamine (10 μM, $n = 11$ neurons) prevents inward currents initiated by Oxo-M ($n = 35$ neurons).
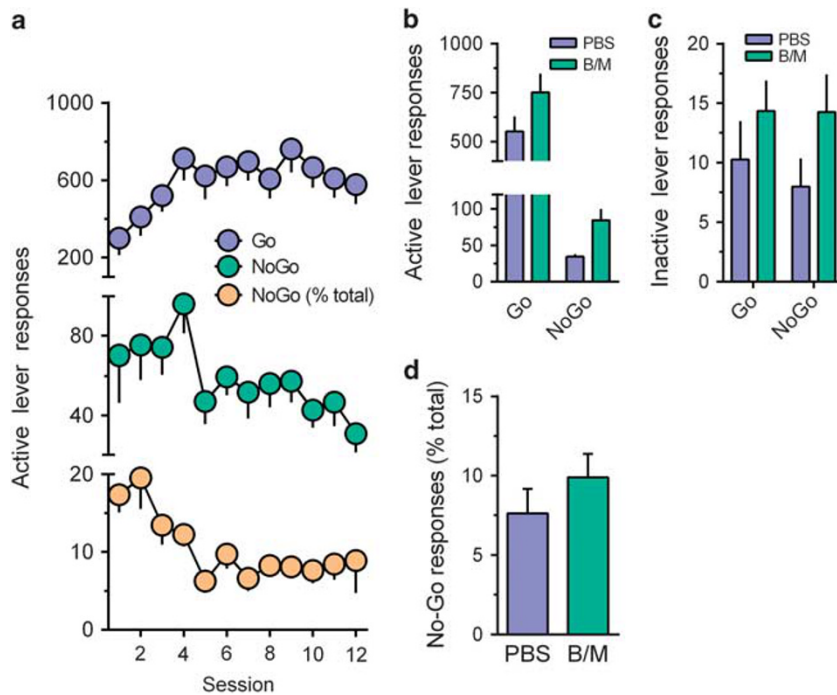
Figure 5 Acquisition of the discrimination between reinforced and non-reinforced intervals in a Go–NoGo task in rats responding for sucrose (a). Effects of LHb inhibition on active (b) or inactive (c) lever responses in a Go–NoGo task for sucrose. Effects of LHb inhibition on the percentage of active lever responses during the NoGo intervals. All data are mean ± SEM ($n = 11$).

neurons demonstrated either biphasic outward–inward I_{hold} responses to Oxo-M (34.4%; Figure 4a and b), consistent with initial hyperpolarization followed by depolarization of the membrane potential, or they showed monophasic outward I_{hold} responses (9.4%), consistent with hyperpolarization (Figure 4a and b). The remaining neurons showed no response to Oxo-M (6.2%, Figure 4a and b). In additional experiments, the depolarizing outward currents were completely blocked by the mACh receptor antagonist scopolamine (10 μM; Figure 4c). Collectively, these data indicate that the majority (84.4%) of LHb neurons are depolarized by mACh receptor activation, and this may account for the ability of scopolamine to disrupt NoGo responding for cocaine *in vivo*.

Experiment 5. LHb Inhibition Does Not Impair Go–NoGo Responding for Sucrose Reinforcement

To determine whether disrupted Go–NoGo performance by LHb inactivation is specific to responding for cocaine, we examined its effect in animals trained in a Go–NoGo task for sucrose reward. Rats significantly decreased the percentage of non-reinforced sucrose-seeking responses during NoGo intervals from 17.4% of total responses to 9.0% over 12 sessions ($F_{(11,110)} = 3.64$, $p = 0.021$, one-way ANOVA, Figure 5a). However, LHb inhibition with B/M did not significantly alter the number of responses during NoGo periods in these sucrose-trained animals (main effect of LHb infusions, $F_{(1,10)} = 3.7$, $p = 0.08$, two-way ANOVA, Figure 5b). Moreover, there was also no change in responding during Go intervals during these infusions (Go–NoGo interval × LHb infusion interaction, $F_{(1,10)} = 1.92$, $p = 0.19$, two-way ANOVA, Figure 5b). As a result, LHb inactivation

did not significantly alter the percentage of responses during the NoGo intervals (PBS = 7.6, 4.2 - 11.1; B/M = 9.9, 6.6–13.9, mean and 95% confidence intervals; $t_{(10)} = 1.37$, $p = 0.20$, paired *t*-test, Figure 5d). There was also no effect of LHb inactivation on inactive lever responses (main LHb infusion effect, $F_{(1,10)} = 4.24$, $p = 0.07$, two-way ANOVA, Figure 5c), and no interval selectivity (Go–NoGo interval × LHb infusion interaction, $F_{(1,10)} = 0.44$, $p = 0.52$, non-significant, two-way ANOVA, Figure 5c). Therefore, unlike Go–NoGo responding for cocaine, LHb inhibition did not alter this discrimination for sucrose reinforcement.

DISCUSSION

Here we examine involvement of the LHb in the control of cocaine seeking using several behavioral models. LHb inhibition did not alter the motivation to seek and self-administer cocaine measured by PR schedule break points nor with seeking responses in a seeking/taking chained schedule of cocaine reinforcement. Similarly, LHb inhibition did not alter punished suppression of cocaine seeking using foot shock administered either after the seeking response or during receipt of the drug. This distinction is important as coincident delivery of reward and punishment can diminish the effectiveness of punishment (Dickinson and Pearce, 1976). As the LHb is involved in behavioral responses to negative outcomes, prediction errors (Hong et al, 2011; Matsumoto and Hikosaka, 2007, 2009), and other aversive events (Stamatakis and Stuber, 2012), the lack of an effect of LHb inhibition on punished responding for cocaine in our study is somewhat surprising. However, our results agree with another study demonstrating that the LHb is not

is predicted by early increased responding when the drug is unavailable (Deroche-Gamonet and Piazza, 2014). In this context, our findings suggest that the LHb is involved in the withholding of responses for cocaine and that experience with this drug diminishes this control (de Wit, 2009). However, the inability to withhold responses following LHb inhibition does not appear to extend to sucrose self-administration, suggesting an impairment associated specifically with cocaine seeking. In this regard, we found that rats responding for sucrose acquired the discrimination between Go and NoGo periods more quickly than those trained for cocaine (compare Figure 1f and Figure 5a), as they display much lower responding during initial NoGo intervals (17% NoGo responses for sucrose vs 51% for cocaine). Moreover, the sucrose-trained animals maintain lower levels of responding during NoGo intervals after the task has been learned (7–9% vs 15–20%, respectively). This is consistent with a greater difficulty in withholding responses for cocaine vs sucrose. This disparity may be related to prior exposure to cocaine, as earlier studies show that the bundle of LHb efferent axons known as the fasciculus retroflexus degenerates after high-dose, extended access psychostimulant exposure (Ellison, 1992; Lax et al, 2013) and that LHb metabolic activity decreases following acute and chronic exposure to cocaine (Hammer and Cooke, 1994; London et al, 1986). Thus it is possible that the participation of the LHb in controlling responding for cocaine may be compromised by limited cocaine exposure, such as that used in our study, and that more extensive exposure may lead to further reductions in LHb output to downstream brain areas that are involved in regulating drug seeking. Thus damage to LHb efferents by psychostimulants may limit the inhibitory control that this brain structure exerts over drug intake, thereby driving compulsive drug seeking observed in addiction.

REFERENCES

Anker JJ, Gliddon LA, Carroll ME (2008). Impulsivity on a Go/No-go task for intravenous cocaine or food in male and female rats selectively bred for high and low saccharin intake. *Behav Pharmacol* 19: 615–629.

Anker JJ, Perry JL, Gliddon LA, Carroll ME (2009). Impulsivity predicts the escalation of cocaine self-administration in rats. *Pharmacol Biochem Behav* 93: 343–348.

Baker PM, Oh SE, Kidder KS, Mizumori SJ (2015). Ongoing behavioral state information signaled in the lateral habenula guides choice flexibility in freely moving rats. *Front Behav Neurosci* 9: 295.

Bassant MH, Jazat F, Lamour Y (1993). Tetrahydroaminoacridine and physostigmine increase cerebral glucose utilization in specific cortical and subcortical regions in the rat. *J Cereb Blood Flow Metab* 13: 855–864.

Belin D, Mar AC, Dalley JW, Robbins TW, Everitt BJ (2008). High impulsivity predicts the switch to compulsive cocaine-taking. *Science* 320: 1352–1355.

Belin-Rauscent A, Fouyssac M, Bonci A, Belin D (2016). How preclinical models evolved to resemble the diagnostic criteria of drug addiction. *Biol Psychiatry* 79: 39–46.

Christoph GR, Leonzio RJ, Wilcox KS (1986). Stimulation of the lateral habenula inhibits dopamine-containing neurons in the substantia nigra and ventral tegmental area of the rat. *J Neurosci* 6: 613–619.

Deroche-Gamonet V, Belin D, Piazza PV (2004). Evidence for addiction-like behavior in the rat. *Science* 305: 1014–1017.

Deroche-Gamonet V, Piazza PV (2014). Psychobiology of cocaine addiction: Contribution of a multi-symptomatic animal model of loss of control. *Neuropharmacology* 76 Pt B: 437–449.

Dickinson A, Pearce J (1976). Preference and response suppression under different correlations between shock and a positive reinforcer in rats. *Learn Motiv* 7: 66–85.

Dougherty DM, Bjork JM (2003). Behavioral impulsivity paradigms: a comparison in hospitalized adolescents with disruptive behavior disorders. *J Child Psychiatry* 44: 1145–1157.

Eagle DM, Bari A, Robbins TW (2008). The neuropsychopharmacology of action inhibition: cross-species translation of the stop-signal and go/no-go tasks. *Psychopharmacology (Berl)* 199: 439–456.

Economidou D, Pelloux Y, Robbins TW, Dalley JW, Everitt BJ (2009). High impulsivity predicts relapse to cocaine-seeking after punishment-induced abstinence. *Biol Psychiatry* 65: 851–856.

Ellison G (1992). Continuous amphetamine and cocaine have similar neurotoxic effects in lateral habenular nucleus and fasciculus retroflexus. *Brain Res* 598: 353–356.

Friedman A, Lax E, Dikshtein Y, Abraham L, Flaumenhaft Y, Sudai E et al (2010). Electrical stimulation of the lateral habenula produces enduring inhibitory effect on cocaine seeking behavior. *Neuropharmacology* 59: 452–459.

Gifuni AJ, Jozaghi S, Gauthier-Lamer A-CC, Boye SM (2012). Lesions of the lateral habenula dissociate the reward-enhancing and locomotor-stimulant effects of amphetamine. *Neuropharmacology* 63: 945–957.

Gill MJ, Ghee SM, Harper SM, See RE (2013). Inactivation of the lateral habenula reduces anxiogenic behavior and cocaine seeking under conditions of heightened stress. *Pharmacol Biochem Behav* 111: 24–29.

Good CH, Wang H, Chen Y-HH, Mejias-Aponte CA, Hoffman AF, Lupica CR (2013). Dopamine D4 receptor excitation of lateral habenula neurons via multiple cellular mechanisms. *J Neurosci* 33: 16853–16864.

Hammer RP, Cooke ES (1994). Gradual tolerance of metabolic activity is produced in mesolimbic regions by chronic cocaine treatment, while subsequent cocaine challenge activates extra-pyramidal regions of rat brain. *J Neurosci* 14: 4289–4298.

Herkenham M, Nauta WJ (1977). Afferent connections of the habenular nuclei in the rat. A horseradish peroxidase study, with a note on the fiber-of-passage problem. *J Comp Neurol* 173: 123–146.

Hikosaka O (2010). The habenula: from stress evasion to value-based decision-making. *Nat Rev Neurosci* 11: 503–513.

Hong S, Jhou TC, Smith M, Saleem KS, Hikosaka O (2011). Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. *J Neurosci* 31: 11457–11471.

Jean-Richard Dit, Bressel P, McNally GP (2014). The role of the lateral habenula in punishment. *PLoS One* 9: e111699.

Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC (2009). The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron* 61: 786–800.

Jhou TC, Good CH, Rowley CS, Xu S-PP, Wang H, Burnham NW et al (2013). Cocaine drives aversive conditioning via delayed

activation of dopamine-responsive habenular and midbrain pathways. *J Neurosci* 33: 7501–7512.

Ji H, Shepard PD (2007). Lateral habenula stimulation inhibits rat midbrain dopamine neurons through a GABA(A) receptor-mediated mechanism. *J Neurosci* 27: 6923–6930.

Kha H, Finkelstein D, Pow D, Lawrence A, Horne M (2000). Study of projections from the entopeduncular nucleus to the thalamus of the rat. *J Comp Neurol* 426: 366–377.

Lane SD, Moeller FG, Steinberg JL, Buzby M, Kosten TR (2007). Performance of cocaine dependent individuals and controls on a response inhibition task with varying levels of difficulty. *Am J Drug Alcohol Abuse* 33: 717–726.

Lax E, Friedman A, Croitoru O, Sudai E, Ben-Moshe H, Redlus L et al (2013). Neurodegeneration of lateral habenula efferent fibers after intermittent cocaine administration: implications for deep brain stimulation. *Neuropharmacology* 75: 246–254.

Lecca S, Meye FJ, Mameli M (2014). The lateral habenula in addiction and depression: an anatomical, synaptic and behavioral overview. *Eur J Neurosci* 39: 1170–1178.

Lecourtier L, Defrancesco A, Moghaddam B (2008). Differential tonic influence of lateral habenula on prefrontal cortex and nucleus accumbens dopamine release. *Eur J Neurosci* 27: 1755–1762.

Lecourtier L, Kelly PH (2005). Bilateral lesions of the habenula induce attentional disturbances in rats. *Neuropsychopharmacology* 30: 484–496.

Lecourtier L, Kelly PH (2007). A conductor hidden in the orchestra? Role of the habenular complex in monoamine transmission and cognition. *Neurosci Biobehav Rev* 31: 658–672.

London E, Wilkerson G, Goldberg S, Risner M (1986). Effects of L-cocaine on local cerebral glucose utilization in the rat. *Neurosci Lett* 68: 73–78.

Maroteaux M, Mameli M (2012). Cocaine evokes projection-specific synaptic plasticity of lateral habenula neurons. *J Neurosci* 32: 12641–12646.

Matsumoto M, Hikosaka O (2007). Lateral habenula as a source of negative reward signals in dopamine neurons. *Nature* 447: 1111–1115.

Matsumoto M, Hikosaka O (2009). Representation of negative motivational value in the primate lateral habenula. *Nat Neurosci* 12: 77–84.

McDonald J, Schleifer L, Richards JB, de Wit H (2003). Effects of THC on behavioral measures of impulsivity in humans. *Neuropsychopharmacology* 28: 1356–1365.

Mello Cruz AP, de, Pinheiro G, Alves SHH, Ferreira G, Mendes M, Faria L et al (2005). Behavioral effects of systemically administered MK-212 are prevented by ritanserin microinfusion into the basolateral amygdala of rats exposed to the elevated plus-maze. *Psychopharmacology (Berl)* 182: 345–354.

Meye FJ, Valentinova K, Lecca S, Marion-Poll L, Maroteaux MJ, Musardo S et al (2015). Cocaine-evoked negative symptoms require AMPA receptor trafficking in the lateral habenula. *Nat Neurosci* 18: 376–378.

Moriizumi T, Hattori T (1992). Choline acetyltransferase-immunoreactive neurons in the rat entopeduncular nucleus. *Neuroscience* 46: 721–728.

Murray JE, Belin D, Everitt BJ (2012). Double dissociation of the dorsomedial and dorsolateral striatal control over the acquisition and performance of cocaine seeking. *Neuropsychopharmacology* 37: 2456–2466.

Olmstead MC, Parkinson JA, Miles FJ, Everitt BJ, Dickinson A (2000). Cocaine-seeking by rats: regulation, reinforcement and activation. *Psychopharmacology (Berl)* 152: 123–131.

Paine TA, Dringenberg HC, Olmstead MC (2003). Effects of chronic cocaine on impulsivity: relation to cortical serotonin mechanisms. *Behav Brain Res* 147: 135–147.

Paine TA, Olmstead MC (2004). Cocaine disrupts both behavioural inhibition and conditional discrimination in rats. *Psychopharmacology (Berl)* 175: 443–450.

Pelloux Y, Everitt BJ, Dickinson A (2007). Compulsive drug seeking by rats under punishment: effects of drug taking history. *Psychopharmacology (Berl)* 194: 127–137.

Richardson NR, Roberts DC (1996). Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *J Neurosci Methods* 66: 1–11.

Santini E, Sepulveda-Orengo M, Porter JT (2012). Muscarinic receptors modulate the intrinsic excitability of infralimbic neurons and consolidation of fear extinction. *Neuropsychopharmacology* 37: 2047–2056.

Satoh K, Fibiger HC (1986). Cholinergic neurons of the laterodorsal tegmental nucleus: efferent and afferent connections. *J Comp Neurol* 253: 277–302.

Stamatakis AM, Stuber GD (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nat Neurosci* 15: 1105–1107.

Stopper CM, Floresco SB (2013). What's better for me? Fundamental role for lateral habenula in promoting subjective decision biases. *Nat Neurosci* 17: 33–35.

Sun W, Akins C, Mattingly A, Rebec G (2005). Ionotropic glutamate receptors in the ventral tegmental area regulate cocaine-seeking behavior in rats. *Neuropsychopharmacology* 30: 2073–2081.

Sutherland RJ (1982). The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. *Neurosci Biobehav Rev* 6: 1–13.

Tuerke KJ, Limebeer CL, Fletcher PJ, Parker LA (2012). Double dissociation between regulation of conditioned disgust and taste avoidance by serotonin availability at the 5-HT(3) receptor in the posterior and anterior insular cortex. *J Neurosci* 32: 13709–13717.

Vanderschuren L, Ahmed S (2013). Animal studies of addictive behavior. *Cold Spring Harb Perspect Med* 3: a011932.

Vanderschuren LJMJ, Everitt BJ (2004). Drug seeking becomes compulsive after prolonged cocaine self-administration. *Science* 305: 1017–1019.

Velasquez KM, Molfese DL, Salas R (2014). The role of the habenula in drug addiction. *Front Hum Neurosci* 8: 174.

Vilaró MT, Palacios JM, Mengod G (1990). Localization of m5 muscarinic receptor mRNA in rat brain examined by *in situ* hybridization histochemistry. *Neurosci Lett* 114: 154–159.

Vilaró MT, Wiederhold KH, Palacios JM, Mengod G (1992). Muscarinic M2 receptor mRNA expression and receptor binding in cholinergic and non-cholinergic cells in the rat brain: a correlative study using *in situ* hybridization histochemistry and receptor autoradiography. *Neuroscience* 47: 367–393.

Wickham R, Solecki W, Rathbun L, McIntosh JM, Addy NA (2013). Ventral tegmental area α6β2 nicotinic acetylcholine receptors modulate phasic dopamine release in the nucleus accumbens core. *Psychopharmacology (Berl)* 229: 73–82.

de Wit H (2009). Impulsivity as a determinant and consequence of drug use: a review of underlying processes. *Addict Biol* 14: 22–31.

Zapata A, Minney VL, Shippenberg TS (2010). Shift from goal-directed to habitual cocaine seeking after prolonged experience in rats. *J Neurosci* 30: 15457–15463.

Zhang C-XX, Zhang H, Xu H-YY, Li M-XX, Wang S (2013). The lateral habenula is a common target of cocaine and dexamethasone. *Neurosci Lett* 555: 12–17.

Zuo W, Chen L, Wang L, Ye J-HH (2013). Cocaine facilitates glutamatergic transmission and activates lateral habenular neurons. *Neuropharmacology* 70: 180–189.

Zuo W, Zhang Y, Xie G, Gregor D, Bekker A, Ye J-HH (2016). Serotonin stimulates lateral habenula via activation of the post-synaptic serotonin 2/3 receptors and transient receptor potential channels. *Neuropharmacology* 101: 449–459.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)