Published in final edited form as:

Neuropharmacology. 2010 November ; 59(6): 452–459. doi:10.1016/j.neuropharm.2010.06.008.

Electrical stimulation of the lateral habenula produces enduring inhibitory effect on cocaine seeking behavior

Alexander Friedman, PhD¹, Elad Lax, MSc², Yahav Dikshtein, MSc², Lital Abraham, BSc, Yakov Flaumenhaft, BSc², Einav Sudai, MSc², Moshe Ben-Tzion, PhD³, Lavi Ami-Ad, MSc⁴, Rami Yaka, PhD⁴, and Gal Yadid, PhD^{1,2}

¹Leslie Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center

²The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University

³Department of Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel

⁴The Institute of Drug Research, School of Pharmacy, The Hebrew University of Jerusalem, 91120, Israel

Abstract

The lateral habenula (LHb) is critical for modulation of negative reinforcement, punishment and aversive responses. In light of the success of deep-brain-stimulation (DBS) in the treatment of neurological disorders, we explored the use of LHb DBS as a method of intervention in cocaine self-administration, extinction, and reinstatement in rats. An electrode was implanted into the LHb and rats were trained to self-administer cocaine (21 days; 0.25–1 mg/kg) until they achieved at least three days of stable performance (as measured by daily recordings of active lever presses in self-administration cages). Thereafter, rats received DBS in the presence or absence of cocaine. DBS reduced cocaine seeking behavior during both self-administration and extinction training. DBS also attenuated the rats' lever presses following cocaine reinstatement (5–20 mg/kg) in comparison to sham-operated rats. These results were also controlled by the assessment of physical performance as measured by water self-administration and an open field test, and by evaluation of depressive-like manifestations as measured by the swim and two-bottles-choice tests. In contrast, LHb lesioned rats demonstrated increased cocaine seeking behavior as demonstrated by a delayed extinction response. In the ventral tegmental area, cocaine self-administration elevated glutamatergic receptor subunits NR1 and GluR1 and scaffolding protein PSD95, but not GABA_Aβ, protein levels. Following DBS treatment, levels of these subunits returned to control values. We postulate that the effect of both LHb modulation and LHb DBS on cocaine reinforcement may be via attenuation of the cocaine-induced increase in glutaminergic input to the VTA.

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Corresponding author: Prof. Gal Yadid, Ph.D. Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center Bar-Ilan University Ramat-Gan ISRAEL Phone: 972-3-531-8123 FAX: 972-3-635-4965 yadidg@mail.biu.ac.il.

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Financial Disclosures All the authors state that they have no conflict of interest to declare.

GY (corresponding author) certifies that all authors have agreed to all the content in the manuscript, including the data as presented.

Keywords

addiction; deep brain stimulation; cocaine self-administration; sucrose self-administration; lateral habenula; negative reward

Introduction

Over the years, attempts have been made to elucidate the mechanisms underlying drug addiction. Cocaine addiction is characterized by progressive increases in drug intake over time, suggesting maladaptive changes in motivational and reward systems. Under normal circumstances this system, known as the mesolimbic dopaminergic system, is in charge of rewarding adaptive behaviors such as food intake and sex. Dopaminergic neurons in the ventral tegmental area (VTA) play a critical role in motivated goal-seeking behavior and reward (Koob and Bloom, 1988; Le Moal and Simon, 1991). The mesolimbic system has also been implicated as a significant component in the rewarding effects of drug usage. The assumption is that drugs of abuse usurp the brain's natural reward system (Everitt et al., 2001; Kauer and Malenka, 2007). Cocaine also increases AMPA and NMDA receptor-mediated glutamatergic transmission in VTA dopaminergic neurons (Schilstrom et al., 2006; Argilli *et al.* 2008, Schumann *et al.* 2009, Schumann & Yaka 2009). VTA glutamatergic inputs are mainly from the medial prefrontal cortex (mPFC) and lateral amygdala with some input from other brain regions (Carr and Sesack, 2000). The change in glutamate induced by cocaine can, in turn, modulate dopamine release in the mPFC and nucleus accumbens (NAc). Even a single exposure to cocaine in naive animals is sufficient to trigger sustained changes on VTA glutamatergic synapses that resemble activity-dependent long-term potentiation (LTP) (Ungless et al., 2001). Synaptic plasticity in the VTA has been implicated in the acquisition of a drug-dependent state. Indeed, chronic cocaine administration was shown to increase AMPA receptor glutamate receptor 1 (GluR1) subunit in the VTA (Carlezon and Nestler, 2002; Fitzgerald et al., 1996). Lasting changes in AMPA receptor-mediated glutamatergic transmission in VTA dopaminergic neurons are thought to contribute to the development of addiction (Carlezon and Nestler, 2002; Wolf, 2003). In light of this theory, it seems that in order to intervene in the addiction process it might be useful to adjust the glutaminergic input to the VTA, as part of the reward system's chain of events that enables addiction to occur.

The lateral habenula (LHb) innervates the VTA and prefrontal cortex with glutaminergic neurons (Lecourtier and Kelly, 2007; Omelchenko et al., 2009). This glutamatergic LHb projection to the VTA was previously suggested to synapse not on dopamine-containing neurons but on GABA interneurons (Lecourtier and Kelly, 2007). However, a recent report suggests that the robust inhibition of dopaminergic cells evoked by the LHb is unlikely to arise from a selective innervation of VTA GABAergic neurons. Moreover, the LHb was suggested to mediate a direct excitation of dopaminergic cells that is over-ridden by indirect inhibition originating from an extrinsic source (Omelchenko et al., 2009).

In one study (Lisoprawski et al., 1980), lesions to the LHb led to increases in dopamine utilization in the prefrontal cortex. Additional studies discovered that electrical stimulation of the LHb inhibits the firing of up to 97% of the dopaminergic neurons in the substantia nigra compacta and the VTA (Christoph et al., 1986; Ji and Shepard, 2007). These findings lead Hikosaka and colleagues to suggest the potential ability of the LHb to influence reward processes (Hikosaka et al., 2008). Recordings of LHb and dopaminergic neurons in the brain of rhesus monkeys were taken during performance of a visual task with positionally biased reward outcomes. The strongest LHb excitation was displayed when a no-reward event was preceded by a reward prediction cue (Matsumoto and Hikosaka, 2007). This, taken together with the finding that stimulation of the LHb results in inhibition of dopamine neurons, led the

researchers to propose that the LHb is involved in negative reward processes, i.e. processes which encode situations where no reward is available (Matsumoto and Hikosaka, 2007). However, the above results demonstrated response of the LHb to natural rewards and not to substances of abuse. Cocaine and other drugs of abuse have been shown to induce selective degeneration of the fasciculus retroflexus that project from the habenula to mesolimbic regions (Ellison, 2002). Nonetheless, no data exist as to the effect of the LHb on the VTA during the period in which chronic cocaine use induces a long lasting impact on reward system plasticity.

Electrical deep-brain-stimulation (DBS) is a technique used to treat and alleviate symptoms of a number of neurological disorders (Kern and Kumar, 2007). Habenular DBS was successfully used in one patient with major depression (Sartorius and Henn, 2007). In the current study, we examined the influence of both LHb stimulation and LHb lesions on cocaine self-administration. We found that LHb stimulation significantly attenuated cocaine-seeking behavior during maintenance, and promoted an extinction response. In contrast, lesions of the LHb did not affect the maintenance phase, but resulted in a delayed extinction response and ultimately prevented attainment of the extinction criterion for cocaine seeking behavior (i.e., a 20% decrease in active lever presses as compared to the first day of cocaine extinction). Because anhedonia and reduced motivation are the core symptoms of depressive-like behavior, we measured these parameters to control for the possibility of inducing general depression by DBS. In addition, cocaine increased VTA protein levels of glutamatergic receptor subunits NR1 and GluR1, and of the scaffolding protein PSD95. DBS of the LHb was associated with normalization of these protein levels.

Materials and Methods

Animals

Male (250–300 g) Sprague Dawley rats were maintained under conditions of unvarying temperature (25°C) and humidity (50%), in a 12:12h light/dark cycle, with free access to food and water. All animal procedures were approved by the Bar-Ilan University Animal Care Committee and were carried out in accordance with the U.S. National Research Council Guide for the Care and Use of Laboratory Animals.

Guide-cannula implantation for lesion

Rats were anesthetized with Ketamine hydrochloride (100 mg/kg, I.P.) and Xylazine (10 mg/kg, I.P.), prior to stereotaxic surgery. A guide-cannula (Plastic One, 30 gauge) was inserted into the LHb (anterior –3.8, lateral –0.9, ventral –3.8 mm from bregma) and sealed by a cannula-dummy (Plastics One). The implantation was secured to the skull with screws and dental acrylic cement. Carprofen (2 mg/kg, I.P.) was injected post-surgery. All lesions were to be performed unilaterally, in the right hemisphere.

DBS electrode construction and implantation

Animals were anesthetized with Ketamine hydrochloride (100 mg/kg, I.P.) and Xylazine (10 mg/kg, I.P.) prior to stereotaxic surgery. A bipolar self-designed stimulating electrode (2 stainless steel electrodes, 0.01 mm diameter, 1 mm between cathode and anode, non-isolated electrode tip 1 mm) was inserted into the brain in such a way that the LHb was situated between the cathode and anode electrodes (cathode/anode anterior –3.8/–3.8, lateral –1.4/–2.4, ventral 4.74/4.74 mm from bregma, with 14° angle). The electrode was secured to the skull with screws and dental acrylic cement. Carprofen (2 mg/kg, I.P.) was injected post-surgery. All stimulations were to be performed unilaterally, in the right hemisphere.

Intravenous catheterization

Following anesthetization, the rats that were to be subjects of the cocaine seeking experiments were also implanted with intravenous Silastic catheters (Dow Corning, Midland, MI) into the right jugular vein (Roth-Deri et al., 2003). The catheter was secured to the vein with silk sutures and was passed subcutaneously to the top of the skull where it exited into a connector (a modified 22 gauge cannula; Plastics One, Roanoke, VA) that was mounted to the skull with MX-80 screws (Small Parts, Inc., Miami Lakes, FL) and dental cement (Yates and Bird, Chicago, IL).

Cocaine self-administration

Ten days after catheterization, rats were transferred daily into the operant conditioning (self-administration) chambers (Med-Associates, Inc.; St Albans Vermont) during their dark cycle, and trained to self-administer cocaine (obtained from the National Institute on Drug Abuse, Research Technology Branch, Rockville, MD, USA) in 60 min sessions. Each self-administration chamber (30×25×22 cm) had two levers, active and inactive, located 5 cm above the floor of the chamber. The self-administration chambers and the computer interface were built locally and were controlled by a computer program written by Steve Cabilio (Concordia University, Montreal, PQ, Canada). An active lever press generated a cocaine infusion (i.v., 0.13 ml/0.25-1mg/kg/5 sec) through the catheter which was connected to an injection device. Throughout cocaine infusion intervals, a light located above the active lever was lit for 20 sec, 15 sec beyond the cocaine infusion period which lasted only 5 sec. During the 15-sec intervals, active lever presses were recorded, but no additional cocaine reinforcement was provided. Rats were trained by an FR1 schedule for up to 10–12 days until stable maintenance levels were attained (at least 3 days with <20% variation in the number of active lever presses). Presses on inactive levers were recorded, but they did not activate the infusion pump and light. Rats were returned to home cages at the end of the daily session.

Lesion procedure

After reaching post-operation maintenance of cocaine intake, a cannula (Plastics One) with a 1 mm projection was inserted into the guide-cannula. Quinolinic acid (Sigma Chemicals) was dissolved in 1 N NaOH and diluted with phosphate-buffered saline (PBS) to a final pH of 7.4 and concentration of 120 nmol/μl. This solution was infused through the cannula using an electronic driven pump (Kopf, microinjection unit, model 5000) for 6 min, to a total amount of 1.2μl. Following infusion of the quinolinic acid, the guide-cannula was sealed by a cannula-dummy (Plastics One) in order to reduce upward diffusion of the solution.

Electrical stimulation procedure

Animals received DBS for 15 min, after being transferred to the self-administration chambers. The parameters for the DBS were 200 μ-amperes, with a spike-duration of 0.5 msec. In a preliminary study, we continually adjusted the stimulation pattern, in order to achieve a reduction in the number of active lever presses within a sucrose-seeking task. We found that low frequency stimulation (10 Hz) elevated the number of presses (120% over baseline), while high frequency stimulation (100 Hz) had no effect at all. We discovered that a specific combination of alternating low (10 Hz) and high frequency stimulations (100 Hz) achieved an optimal reductive effect on lever presses. Therefore we decided to employ a combined stimulation that was constructed from four different sets of patterns: 1) Low frequency stimulation of 10 HZ, 2) High frequency stimulation of 100 Hz, 3) Low-frequency burst stimulation (180 msec intervals between bursts; within each burst there were 5 spikes with 80 msec intervals between spikes). 4) High-frequency burst stimulation (300 msec intervals between bursts; within each burst there were 30 spikes with 3 msec intervals between spikes). Alterations between high and low frequency patterns were performed constantly. The duration

of high frequency stimulation was (max) 4 sec per stimulation, with 20 second-long pauses between stimulations (in order to avoid tissue damage). The duration of low frequency stimulation was between 15 and 60 sec.

Extinction procedure

After reaching stable maintenance levels of cocaine-seeking, rats were again placed in the operant conditioning chambers for 1 hour, this time with no cocaine available. This procedure was continued daily (for up to 6–7 days), until rats reached the extinction criterion, i.e. 3 days of 20% decrease in active lever presses as compared to the first day of cocaine extinction.

Reinstatement procedure

One day after reaching the extinction criterion, rats received a single IP injection of cocaine in the self-administration cages. Because some of the rats had plugged catheters at the time of reinstatement, all rats received an IP, and not IV, injection of cocaine. Cocaine was injected in increasing doses, on consecutive days, as follows: 0 mg/kg; 5 mg/kg; 10 mg/kg; 15 mg/kg and 20 mg/kg (0.4 ml, each dose). Injections were combined with a light-cue. The rats' response in the self-administration cages was subsequently monitored.

Forced Swim Test

Rats were placed in a cylindrical tank (40 cm high and 18 cm in diameter) containing enough water (at 2°C higher than room temperature) to allow rats to touch the bottom of the tank with their tail. Immobility of rats was then measured. Immobility was defined as suspension of swimming, in such a manner that both hind paws were immobile. Test duration was 5 minutes (Dremencov et al., 2006; Overstreet, 1993; Overstreet et al., 2005).

Two bottle choice test

Two bottles were inserted into the rats' home cage. One bottle contained water while the other contained a mixture of water and sucrose (4%). The levels of sucrose/water consumption were measured for 12 h (Bechtholt et al., 2008).

Preparation of brain homogenates and Western blot analysis

Following the behavioral tests, randomly selected rats were anaesthetized with isoflurane and decapitated. Brains were rapidly removed, and the VTA was dissected bilaterally on an ice-cold platform and immediately transferred to liquid nitrogen in Eppendorf tubes. Tissue was homogenized in homogenization buffer (HB) [320 mM sucrose, 10mM Tris-HCl, pH 7.4, 1mM EDTA, 1mM EGTA, protease inhibitor cocktail (Sigma, St. Louis, MO)]. Homogenates were centrifuged at 1000 g for 5 min at 4°C to remove nuclei and large debris and the supernatant was kept for further analysis. Protein (50 mg) from total brain homogenates was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4°C with anti-GluR1 (Millipore Bioscience Research Reagents, Billerica, MA), anti-NR1 (Zymed, SF, CA), anti-GluR1 (Chemicon, Temecula, CA), anti-PSD95 (Millipore), bd19 monoclonal anti-GABAA receptor β 2, 3 chain (Chemicon, Temecula, CA) and anti-Tubulin (Sigma, St. Louis, MO) antibodies, and then incubated (1 hr at room temperature) with appropriate Infrared-Dyes (IRDye) conjugated fluorescent secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA). IRDye conjugates are optimized for the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The bands were quantitatively analyzed by densitometry, with NIH Image providing peak areas, and values were expressed as the percentage of control.

Histology

At the conclusion of experiments, randomly selected animals were anesthetized and transcardially perfused with PBS×1 followed by 4% paraformaldehyde. The brains were removed and immersed in 4% paraformaldehyde for 24 hrs, and then in a phosphate buffer with 30% sucrose for 48 hrs. The brains were then frozen on dry ice and sliced (40 µm sections) using a cryostat microtome. Sections were mounted on glass slides (coated with 2% gelatin), stained with Cresyl violet and subsequently examined under a microscope to verify the placement of the electrode (see supplement Fig. 1).

Water self-administration

A separate group of rats were trained to self-administer water in self-administration chambers (Med-Associates, Inc.; St Albans Vermont) during their dark cycle, in 60-min sessions. Rats were allowed 15 ml of water per day in addition to approximately 4.0 ml of water consumed during the daily self-administration sessions. The operant chambers, reinforcement schedule, and session duration were identical to those used for cocaine self-administration. Rats received 0.13 ml of water per active lever press, delivered into a drinking dish in the operant chamber. Responses were monitored in the naive state, following electrode implantation, and after LHB DBS.

Locomotor activity test

A separate group of rats were initially habituated to an open field consisting of a plastic polymer box (90×90×30 cm), 5 min/day. Prior to and after electrode implantation surgery, a camera was placed above the box, and each rat was filmed in the open field for 5 min, so that naive behavioral parameters could be measured. The incidence of line crossing with both hind paws and freezing was recorded. All measurements were performed between 8:00 and 14:00 in red light. The video and computer equipment was situated in a separate room, in which all video and observation analyses were conducted (Janssen et al., 1960; Malkesman et al., 2007; Strekalova et al., 2004).

Experimental Design (see supplemental Figure 2)

Experiment 1: Effect of LHB lesioning on the extinction of cocaine seeking behavior

A guide-cannula was implanted in the LHB, and rats were then trained to self-administer cocaine (0.5 mg/kg, 0.13 ml/infusion). On day 5 of stable maintenance (3 days of <20% variation in number of active lever presses), one group of rats (n=12) underwent chemical lesioning of the LHB, and the control group received vehicle (n=12). The next day, cocaine self-administration was measured for 3 consecutive days in 12 rats from each group. Concurrently, 12 rats from the lesioned and control groups were denied cocaine in the self-administration cages. Extinction of cocaine-seeking behavior was measured daily, for 6 days (for experimental scheme, see supplemental Fig. 2).

Experiment 2: Effect of DBS of the LHB on cocaine seeking behavior

An electrode was implanted in the LHB, and rats were then trained to self-administer cocaine (0.5 mg/kg, 0.13 ml/infusion). On day 5 of stable maintenance, rats were again placed in the self-administration chambers. One group of rats (N=12) received low frequency DBS (10 Hz, 15 min), and a second group (N=12) received high frequency DBS (100 Hz, 15 min). The third group consisted of 3 sub-groups (N=10 in each sub-group) trained to self-administer a dose of either 0.25, or 0.5, or 1mg/kg cocaine, respectively (0.13 ml/infusion per each dose). These rats received the combined set of DBS (15 min). Active lever presses were monitored in all groups. On the next day, the three sub-groups again received combined pattern DBS (15 min), but with no cocaine available. The rats continued daily cocaine extinction sessions, and when

they reached the extinction criterion, they underwent reinstatement (for experimental scheme, see supplemental Fig. 2).

Experiments 2a and 2b: Control of non-specific effects on operant performance

—To rule out non-specific effects of DBS on operant performance, we tested the effect of DBS in a water self-administration test and a locomotor activity test, in separate groups. For water self-administration, rats (N=10) were allowed to achieve 8 days of stable maintenance levels of water self-administration, and an electrode was then implanted into the LHb. Rats' performance was monitored for two weeks after the operation until it was established that post-operation task performance reached that of the pre-operation naive state. Rats then received combined pattern DBS to the LHb (15 min), immediately after the animals were placed in the self-administration cages. The level of water administration was measured daily, for 5 days.

Another group of rats (N=10) were habituated to the open field for 5 min/day, for 14 days. On day 15, each rat was filmed in the open field for 5 min, to measure naive parameters. On day 16, rats underwent electrode implantation into the LHb and were allowed to recover for 14 days. Rats were filmed on day 15 after surgery, and again immediately following combined pattern DBS treatments.

Experiment 3: Effects of combined pattern DBS of the LHb on depressive-like traits

A. Two bottle choice test—At the conclusion of experiment 2, rats randomly selected from the combined pattern DBS (n=10) and levels of sucrose/water consumption were measured in the rats' home cage for 12 h.

B. Forced swim test—Directly after the two bottle choice test, rats randomly selected from the combined pattern DBS (n=10) and sham-operated groups were placed in a cylindrical tank, and immobility was measured for 5 min.

Experiment 4: Protein levels in the VTA after DBS

At the conclusion of experiment 3, rats were randomly selected from treatment and control groups. VTA protein levels of the NR1 subunit of the NMDA receptor, the GluR1 subunit of the AMPA receptor, the scaffolding protein PSD95 and the β chain of the GABA_A receptor were measured using Western blot analysis. Results in DBS-treated rats which self-administered cocaine were compared to sham-operated rats, sham-operated rats which self-administered cocaine, rats treated with DBS alone, and naive untreated rats.

Additionally, rats from the LHb-lesioned groups, and from the low-frequency, high-frequency, and combined pattern DBS groups, were randomly selected at the conclusion of behavioral experiments. Their brains were removed and examined using Cresyl violet staining to verify the accurate placement of the cannula or electrode.

Statistics—All data are expressed as mean $\pm$ standard error of the mean (SEM). Significance was determined by analyses of variance (ANOVA) with repeated measures (days) to examine the effect of the experimental procedures on active lever presses in the cocaine self-administration task. ANOVAs were followed by Student–Newman–Keuls post hoc tests to determine which treatment groups were altered, and $p < 0.05$ was considered significant. Significance between two groups was determined by Student's t-tests.

Results

Experiment 1: Effect of LHb lesion on the extinction of cocaine-seeking behavior

Rats which reached stable maintenance levels of cocaine self-administration underwent LHb lesioning. We found that lesion of the LHb did not affect the maintenance phase of cocaine self-administration (mean number of active lever presses for 3 days in sham operated rats: 22 ± 3 , vs. LHb lesioned rats: 23 ± 3.1). Next, the extinction response of the lesioned group was compared to that of sham-operated rats. We found that the lesioned group failed to reach the extinction criterion. Thus, lesioning of the LHb prevents attainment of a full extinction response. A repeated measures ANOVA for the number of active lever presses during the extinction process revealed a main effect of group [$F(1,139)=70$; $p<0.0001$], a main effect of days [$F(6,139)=10.91$; $p<0.0001$], and an interaction of group and days [$F(6,139)=5.12$; $p<0.04$]. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and lesioned group ($p=0.01$, for days 3–6) (Fig. 1). Finally, the relapse response was examined following cocaine reinstatement. No differences were observed in the number of active lever presses between lesioned and sham-operated rats.

Experiment 2: DBS of the LHb

Rats which reached stable maintenance levels of cocaine self-administration received LHb DBS immediately after being placed in the self-administration cages. The group that received low frequency DBS treatment displayed a significantly higher number of active lever presses compared to those of sham-operated control rats (i.e., with electrodes implanted into the same brain area, but no electrical stimulation given) (Fig. 2A, $F(1,23)$; $p<0.001$, Student's t-test). The group that received high frequency DBS treatment showed a similar number of active lever presses compared to those of sham-operated rats (Fig. 2B).

The three sub-groups that received combined pattern DBS exhibited a significantly lower number of active lever presses compared to those of sham-operated control rats. A one-way ANOVA for the number of active lever presses revealed a main effect of group ($F[2, 27] = 37.94$; $p<0.0001$). A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and DBS-treated groups for all three cocaine doses ($p<0.001$; Fig. 3 A–C). During the 15 minutes of stimulation, the number of lever presses was approximately 4 ± 1 presses.

On the following day, the three sub-groups began the extinction process. Rats once again received combined pattern DBS in the self-administration cages, this time in the absence of cocaine. All groups similarly responded in a significantly lower number of active lever presses compared to the control group. This implies that stimulation of the LHb attenuates the rewarding effects of cocaine, thus effectively inducing extinction of cocaine self-administration behavior. A repeated measures ANOVA for the number of active lever presses revealed a main effect of DBS on self-administration during extinction, as follows: For the sub-group which was trained to self-administer 0.25 mg/kg cocaine, there was a main effect of group [$F(1,112)=15$; $p<0.001$], a main effect of days [$F(6,112)=6.21$; $p<0.001$], and an interaction between group and days [$F(6,121)=0.4$; $p<0.04$] during the extinction process. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and DBS-treated groups ($p=0.001$, for days 1–3) (Fig. 3D). For the sub-group which was trained to self-administer 0.5 mg/kg cocaine, there was a main effect of group [$F(1,112) = 70$; $p<0.0001$], a main effect of days [$F(6,112)=10.91$; $p<0.0001$], and an interaction between group and days [$F(6,112)=5.12$; $p<0.04$] during the extinction process. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and DBS-treated groups ($p=0.001$, for days 1–2) (Fig. 3E). For rats which was trained to self-administer 1 mg/kg cocaine, there was a main effect of group [$F(1,112)=14$; $p<0.0001$], a main effect of

days [$F(6,112)=10.91$; $p<0.0001$], and an interaction between group and days [$F(6,112)=5.12$; $p<0.04$] during the extinction process. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and DBS-treated group ($p=0.001$, for days 1–3) (Fig. 3F).

When the rats reached the extinction criterion (20% decrease in active lever presses as compared to the first day of extinction), they underwent cocaine reinstatement. Cocaine was injected IP at increasing doses (0 mg/kg; 5 mg/kg; 10 mg/kg; 15 mg/kg and 20 mg/kg; 0.4 ml) on consecutive days, combined with a light-cue. The sub-group that was trained to self-administer 0.25 mg/kg cocaine demonstrated a significantly lower number of active lever presses in the reinstatement phase, compared to the control group. A repeated measures ANOVA for the number of active lever presses revealed a main effect of group [$F(1,95)=15$; $p<0.0001$], a main effect of dose [$F(4,29)=7.3$; $p<0.0001$], and an interaction between group and dose [$F(4,95)=3.11$; $p<0.01$]. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and the DBS-treated group: $p<0.001$ for the dose of 10 mg/kg; $p<0.001$ for the dose of 15 mg/kg; and $p<0.001$ for the dose of 20 mg/kg (see Fig. 3G). The second sub-group that had been trained to self-administer 0.5 mg/kg of cocaine also demonstrated a significantly lower number of active lever presses in the reinstatement phase, as compared to the control group. A repeated measures ANOVA for the number of active lever presses revealed a main effect of group [$F(1,95)=45$; $p<0.0001$], a main effect of dose [$F(4,95)=22.3$; $p<0.0001$], and an interaction between group and dose [$F(4,95)=4.03$; $p<0.01$]. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and DBS-treated group: $p<0.001$ for the dose 10 mg/kg; $p<0.001$ for the dose of 15 mg/kg; and $p<0.001$ for the dose of 20 mg/kg (see Fig. 3H). The third sub-group which had been trained to self-administer 1 mg/kg cocaine similarly demonstrated a significantly lower number of active lever presses during the reinstatement phase, compared to the control group. A repeated measures ANOVA for the number of active lever presses revealed a main effect of group [$F(1,95)=19$; $p<0.0001$], a main effect of dose [$F(4,95)=12.13$; $p<0.0001$], and an interaction between group and dose [$F(4,95)=1.22$; $p<0.01$]. A Student-Newman-Keuls post-hoc test showed significant differences between the sham-operated and DBS-treated group: $p<0.001$ for the dose of 10 mg/kg; $p<0.001$ for the dose of 15 mg/kg and $p<0.001$ for the dose of 20 mg/kg (see Fig. 3I).

In order to examine whether the decrease in cocaine self-administration was due to a decrease in the reinforcing effects of cocaine or, possibly, to a generalized decrease in instrumental response, we measured the effect of combined pattern DBS on the water self-administration paradigm. We found that the group which received combined pattern DBS treatment showed no difference in the number of active lever presses (58 ± 5 , average of 5 days response) compared to those of sham-operated rats (60 ± 3 , average of 5 days response). Locomotor activity, as measured by the distance crossed in an open field apparatus, showed no difference between combined pattern DBS-treated rats ($20.09\text{m}\pm3.18\text{m}$) and sham-operated rats ($18.79\text{m}\pm2.16\text{m}$). These findings rule out a non-specific effect of DBS on operant performance.

Experiment 3: Effect of combined pattern DBS of the LHb on depressive-like manifestations

We conducted an anhedonia (two bottle choice) test and a motivational (swim) test, to control for the above findings which demonstrated an attenuating effect of combined-pattern DBS on the rewarding effects of cocaine. No significant differences were found between the sham-operated and DBS-treated groups (Fig. 4A–B, $F(1, 12)=2.11$, $p>0.3$ for two bottle choice test and $F(1, 12)=3$, $p>0.32$ for swim test).

Experiment 4: GluR1 levels in the VTA after DBS

At the end of experiment 3, brains were removed and the VTA was isolated and homogenized. We then measured protein levels of the NR1 subunit of the NMDA receptor, the GluR1 subunit of the AMPA receptor, the scaffolding protein PSD95, and the $\beta 2$ and $\beta 3$ subunits of the GABA_A receptor. NR1, GluR1 and PSD95 levels, but not GABA_A β levels, were significantly increased in sham-operated rats trained to self-administer cocaine. However, in rats trained to self-administer cocaine and treated with combined pattern DBS, expression levels of these proteins returned to baseline control levels (Fig 5; significance was detected by one way ANOVA $F[6, 24]=18.6$ $p<0.0001$, with Student-Newman-Keuls post-hoc). We observed no changes in protein levels of rats treated with DBS alone. This data suggests that DBS can specifically reverse the upregulation of the glutamatergic system in VTA neurons.

Discussion

The findings presented in this study demonstrate that alterations in the normal functioning of the LHb, induced through lesioning and electrical stimulation, affect incentive motivation in drug-related reward processes.

Chemical lesions of the LHb prevented the rats from reaching the defined extinction criterion for cocaine-seeking behavior when cocaine was eliminated. A possible explanation for these results is that the chemical lesions abrogated the inhibitive effect of the LHb on the response of dopaminergic cells to no-reward stimuli (Christoph et al., 1986). This, in turn, prevented the rats from distinguishing between rewarding stimuli (e.g. cocaine) and non-rewarding stimuli.

Previous data showed that synaptic function in VTA dopaminergic neurons is readily but reversibly enhanced by natural reward-seeking behavior, while voluntary cocaine self-administration induces a persistent synaptic enhancement that is resistant to behavioral extinction (Chen et al., 2008). Changes in synaptic strength on VTA dopaminergic neurons are thought to play a major role in the development of addiction-related behaviors. Excitatory synapses onto dopamine neurons of the VTA represent a critical site of psychostimulant-induced synaptic plasticity. Previously it was demonstrated both *in vitro* and *in vivo* that repeated cocaine injections elicit LTP in the VTA and NAc (Argilli et al., 2008; Schumann et al., 2009; Schumann and Yaka, 2009). These changes were associated with an increase in the GluR1 subunit of the AMPA receptor (Carlezon and Nestler, 2002). Moreover, overexpression of GluR1 in the VTA is sufficient to produce behavioral sensitization (Carlezon et al., 1997). We demonstrated that DBS of the LHb in rats trained to self-administer cocaine restored the machinery that sub-serves the LTP process (i.e., the glutamatergic elements NR1, GluR1 and PSD95), to normal control levels. This change is specific to the excitatory glutamatergic system, since no change was found in the GABA_A receptor, as determined by β chain levels of this inhibitory receptor. Therefore, our data suggest that DBS of the LHb weakens cocaine-induced plasticity in the VTA, and provides a potential cellular mechanism by which DBS can affect synaptic plasticity of VTA neurons. This process ultimately leads to reduced cocaine consumption.

The LHb receives inputs mainly from the basal ganglia, several dopaminergic areas such as the VTA, and median raphe serotonergic neurons (Gruber et al., 2007; Lecourtier & Kelly, 2007). The firing frequency of basal ganglia neurons in response to a no-reward prediction cue is approximately 110–150 Hz (Hong & Hikosaka, 2008). Both dopamine and serotonin neurons encode reward-related signals, when excited by reward-predicting targets. Their encoding frequency is approximately 8–12 Hz (Fiorillo et al., 2003; Nakamura et al., 2008). The strongest LHb excitation effect was displayed when a no-reward event was preceded by a reward prediction cue (Hong & Hikosaka, 2008). In the case of forced extinction training, the LHb is

presumably inhibited following a reward prediction cue (a positive stimulus such as a reward-associated context) and strongly activated following a no-reward event, thus producing an extinction response over time. However, this process requires an extended period of time to take effect. It is possible that by use of combined DBS, i.e. applying the specific combination of low and high frequencies signals sequentially, we succeeded in mimicking the electrical inputs received by the LHb from both the basal ganglia and the dopaminergic areas. Thus we effectively accelerated the process of extinction.

A recent paper pointed to the connectivity between the habenulae (left and right hemispheres) via the habenular commissure (Kim, 2009). Indeed, in our study, unilateral stimulation proved to be sufficient in order to attain the desired effect and therefore bilateral stimulation was unnecessary. Similar findings have been reported in treatment of Parkinson's disease, where unilateral stimulation is successful in producing the required effects (Germano et al., 2004). We aimed at manipulating the LHb, while excluding the neighboring medial habenula. However, the medial habenula and LHb are relatively small brain structures. Therefore we cannot rule out incidental lesion and/or stimulation of the medial habenula as well.

In order to substantiate our results, we examined the effect of DBS on an additional protocol, in which the rats were reinstated to cocaine following extinction. Here, too, rats that underwent combined pattern DBS had a lower count of active lever presses as compared to controls. This further demonstrates the attenuating effect of LHb DBS on cocaine-seeking behavior.

It is possible to stipulate that the results of our study stem from the strengthening of LHb functioning. This could lead to reward devaluation, but it could also result in a general depressive-like state, producing similar empirical results. The latter possibility was ruled out by additional experiments, which demonstrated that although DBS-treated, cocaine-trained rats did not respond in an addictive manner when presented with cocaine, they did however continue to consume sucrose to the same extent as sham-operated control rats. Furthermore, their levels of immobility in the swim test were also similar to those of sham-operated control rats. Such behaviors are unlikely to be expected if the rats were indeed suffering from depressive-like symptoms or physical impairments due to the stimulation process (for review see (Friedman et al., 2009; Overstreet et al., 2005)).

The findings of this study, combined with those of previous studies, point to the central role that the LHb plays in the regulation of negative reward responses. As previously mentioned, LHb neurons respond equally to a conditioned stimulus that is paired with a displeasing context, whether it is the absence of reward or the presence of an aversive stimulus (Matsumoto and Hikosaka, 2009). Accordingly, we suggest that the response of rats to LHb DBS may be a consequence either of reward devaluation which signals and directs the rat that previous gratifying circumstances are no longer available, or of an increase in the aversive effects of cocaine. Alternatively, DBS may attenuate reconsolidation of associative-learning memory as suggested previously (Lecourtier et al., 2004). This is in correlation with our own results, which demonstrate that LHb lesions have no effect on self-administration performance when reward is available, and conversely, there is a disability to reach the extinction criterion when cocaine is denied. The finding that stimulation of the LHb resulted in extended effects with regard to reward-seeking behavior is supported by the increase in protein levels of NR1, GluR1 and PSD95 in the VTA.

Similar DBS-based therapies that target other brain regions necessitate constant stimulation in order to uphold the desired effect, such as is the case in the treatment of Parkinson disease, depression and OCD (Germano et al., 2004). In light of this, our findings may have further advantage, because we demonstrate that effective attenuation of cocaine reward requires only short-term DBS of the LHb.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIH (grant # R21-DA027776) to GY. AF was supported by a President's Fellowship, Bar-Ilan University. The research reported in this article was completed as part of AF's Ph.D. dissertation.

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Fig. 1. Effect of LHb lesion on the extinction of cocaine seeking behavior

The extinction processes of chemically lesioned rats and sham-operated control rats were compared. The lesioned group did not reach the extinction criterion, as opposed to control (* $p=0.01$, for days 3–6; repeated measures ANOVA for the number of active lever presses was conducted, followed by a Student-Newman-Keuls post-hoc test). M represents the mean of the last 3 days of maintenance. Mean number of active lever presses is shown for 60 min sessions.

Fig. 2. Effect of low and high frequency DBS of the LHb on cocaine seeking behavior

Rats were trained to self-administer cocaine until reaching stable maintenance levels. Subsequently, one group of rats received low frequency stimulation (**Panel A**), and showed a significantly higher number of active lever presses compared to sham-operated control rats. The other group received high frequency stimulation (**Panel B**), with no effect on cocaine-seeking behavior (* $p < 0.001$, Student's T test for high and low frequency stimulations vs. control). Mean number of active lever presses is shown for 60 min sessions.

Fig. 3.

Panel A–C: Effect of combined pattern DBS of the LHb on cocaine-seeking behavior. Three separate groups of rats were trained to self-administer cocaine (receiving doses of 0.25 mg/kg, 0.5 mg/kg, or 1 mg/kg, respectively) until reaching stable maintenance levels, after which they received combined pattern stimulation. In all three doses, combined pattern stimulation was shown to significantly reduce the number of active lever presses as compared to sham-operated control rats (significance detected by one-way ANOVA for the number of active lever presses, with Student-Newman-Keuls post-hoc; $*p < 0.01$). Panel D–F: Effect of combined pattern DBS of the LHb on the extinction of cocaine seeking behavior. The three groups which were trained to self-administer either 0.25 mg/kg, or 0.5 mg/kg, or 1 mg/kg cocaine again received combined pattern stimulation, but no cocaine. All three groups demonstrated a significantly accelerated extinction process in comparison to the sham-operated control group (significance detected by repeated measures ANOVA and Student-Newman-Keuls post-hoc; $*p < 0.01$). Panel G–I: Effect of combined pattern DBS of the LHb on cocaine reinstatement. When the rats reached the extinction criterion, they underwent reinstatement of cocaine (administered IP on consecutive days, in increasing doses of 0 mg/kg, 5 mg/kg, 10 mg/kg, 15 mg/kg and 20 mg/kg, combined with a light-cue). The three groups that had self-administered three different doses of cocaine during maintenance and had received combined pattern DBS, now demonstrated a significantly lower number of lever presses, as compared to the control group (significance detected using repeated measures ANOVA followed by Student-Newman-Keuls post-hoc for cocaine reinstatement doses; $*p < 0.01$). M represents the mean of the last 3 days of maintenance. Mean number of active lever presses is shown for 60 min sessions.

Fig. 4. Effects of combined pattern DBS of the LHb on depressive-like traits

Possible effects of combined pattern stimulation on depressive-like manifestations were examined using (A) the two-bottle choice test (as a measurement of anhedonia): No significant differences were found between sucrose consumption levels of DBS-treated and sham-operated control rats; and (B) the forced swim test (as a measurement of motivation): No significant differences were found in immobility levels of the DBS-treated rats as compared to sham-operated control rats.

Fig. 5. Levels of NR1, GluR1 and PSD95 in the VTA

Protein levels of the NR1 subunit of the NMDA receptor, GluR1 subunit of the AMPA receptor and scaffolding protein PSD95 were increased following cocaine self-administration. DBS of the LHb in cocaine-trained rats restored NR1, GluR1 and PSD95 levels to normal. This effect was specific to the glutamatergic system, since levels of the GABA_A receptor β subunits (β2 and β3) remained unchanged. DBS alone did not alter the levels of these proteins in the VTA (* $P < 0.001$ cocaine-treated rats vs. control, sham-operated, DBS-treated and cocaine+DBS-treated rats).