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Cocaine facilitates glutamatergic transmission and activates lateral habenular neurons

Wanhong Zuo^{a,b}, Lixin Chen^c, Liwei Wang^{a,*}, and Jiang-Hong Ye^{b,*}

^aDepartment of Physiology, Medical College, Jinan University, Guangzhou, China

^bDepartment of Anesthesiology, Pharmacology and Physiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey, USA

^cDepartment of Pharmacology, Medical College, Jinan University, Guangzhou, China

Abstract

Cocaine administration can be both rewarding and aversive. While much effort has gone to investigating the rewarding effect, the mechanisms underlying cocaine-induced aversion remain murky. There is increasing evidence that the lateral habenula (LHb), a small epithalamic structure, plays a critical role in the aversive responses of many addictive drugs including cocaine. However, the effects of cocaine on LHb neurons are not well explored. Here we show that, in acute brain slices from rats, cocaine depolarized LHb neurons and accelerated their spontaneous firing. The AMPA and NMDA glutamate receptor antagonists, 6, 7-dinitroquinoxaline-2, 3-dione, DL-2-amino-5-phosphono-valeric acid, attenuated cocaine-induced acceleration. In addition, cocaine concentration-dependently enhanced glutamatergic excitation: enhanced the amplitude but reduced the paired pulse ratio of EPSCs elicited by electrical stimulations, and increased the frequency of spontaneous EPSCs in the absence and presence of tetrodotoxin. Dopamine and the agonists of dopamine D1 (SKF 38393) and D2 (quinpirole) receptors, as well as the dopamine transporter blocker (GBR12935), mimicked the effects of cocaine. Conversely, both D1 (SKF 83566) and D2 (raclopride) antagonists substantially attenuated cocaine's effects on EPSCs and firing. Together, our results provide evidence that cocaine may act primarily via an increase in dopamine levels in the LHb that activates both D1 and D2 receptors on the glutamatergic terminals which make synapses on LHb neurons. This leads to an increase in glutamate release and LHb neuron activity, and may contribute to the aversive effect of cocaine observed *in vivo*.

Keywords

Cocaine; Lateral habenula; Glutamate transmission; aversion; dopamine receptors

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*Corresponding Author: J-H Ye, Department of Anesthesiology, UMDNJ, New Jersey Medical School, 185 South Orange Avenue, Newark, New Jersey 07103, USA; Tel: + 1973-972-1866, Fax: +1973-972-4172, ye@umdnj.edu. L-W Wang, Department of Physiology, Medical College, Jinan University, Guangzhou, China; Tel: +86-(0)20-85226565, wangliwei@sohu.com.

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1. Introduction

Cocaine abuse is prevalent throughout the world. Like many other addictive drugs, cocaine has both rewarding and aversive properties (Ettenberg, 2004; Riley, 2011; Serafine et al., 2012a), and a balance between these two effects influences drug abuse potential (Hunt and Amit, 1987). The dopamine transporter (DAT) is a key player in dopamine neurotransmission. Inhibition of the DAT is a major factor that contributes to cocaine reward (Sora et al., 2001). Although recent evidence suggests that dopamine may also contribute to cocaine's aversive property (Freeman et al., 2005; Serafine et al., 2012b), the underlying mechanisms remain murky.

There is increasing evidence that the lateral habenula (LHb), an epithalamic nucleus, plays a critical role in fear, anxiety, stress, drug abuse, and aversion (Bromberg-Martin and Hikosaka, 2011; Matsumoto and Hikosaka, 2007; Pobbe and Zangrossi, 2010; Shabel et al., 2012; Winter et al., 2011). Converging anatomical, physiological, and behavioral studies show that the LHb is one of the few regions of the brain to be inhibited by hedonic stimuli and reward, but activated by various aversive stimuli and reward prediction error (Bromberg-Martin and Hikosaka, 2011; Matsumoto and Hikosaka, 2007). Recent evidence links the LHb to cocaine addiction and aversion. Acute cocaine exposure selectively increases Fos protein expression (an index of neural activity) in the LHb (Zahm et al., 2010; Zhang et al., 2007). Chronic cocaine administration specifically induces degeneration in axons from the LHb to fasciculus retroflexus (Ellison, 2002). Lesions of the LHb increased a delayed cocaine seeking behavior, whereas, deep-brain-stimulation, that depicts glutamate transmission to the LHb, reduced cocaine seeking behavior (Friedman et al., 2010). Cocaine selectively potentiates AMPA receptor-mediated EPSCs in LHb neurons that send axons to GABAergic neurons in the rostromedial tegmental nucleus (RMTg), which send inhibitory projection to the dopamine neurons in the ventral tegmental area (Maroteaux and Mameli, 2012).

The LHb is subject to dopamine regulation. There is mutual innervation between the LHb and the midbrain dopaminergic structures. The LHb not only send their projections (directly and indirectly) to the midbrain dopaminergic neurons but also receive dopaminergic projections (Goncalves et al., 2012; Gruber et al., 2007; Skagerberg et al., 1984). Dopamine activates D1-like (consisting of D1 and D5) and D2-like (D2, D3, and D4) receptor families; both of which play critical roles in cocaine addiction (Friedman et al., 2010; McCulloch et al., 1980; Ramm et al., 1984), and are richly expressed on the LHb (GENSAT, 2008; Savasta et al., 1986; Weiner et al., 1991). Systemic administration of dopamine agonists induces aversive responses such as nausea, vomiting (Perez-Lloret et al., 2010), and conditioned taste aversions (Asin and Montana, 1989), as well as increases firing of LHb neurons and c-fos expression in the LHb. These effects were prevented by the dopamine antagonist haloperidol (Kowski et al., 2009; Wirtshafter et al., 1994). In the current study, we investigated the effects of acute cocaine on the activity and glutamate transmission of the LHb neurons in brain slices of rats.

2. Materials and methods

2.1. Animals and brain slice preparation

All procedures were in accordance with the National Institutes of Health guidelines and with the approval of the Animal Care and Utilization Committee of the University of Medicine and Dentistry of New Jersey. We minimized the number of animals used and their suffering. We studied 70 Sprague-Dawley rats at postnatal days 20–30 (P20–P30). These rats were housed two per cage with food and water available *ad libitum*, and kept on a standard 12-h light/dark cycle with lights on at 7:00 A.M and off at 7:00 P.M. On the experimental days,

rats were deeply anesthetized with ketamine/xylazine (IP) and then decapitated. Brains were rapidly dissected and placed in a cold glycerol-based artificial cerebrospinal fluid (aCSF) containing (in mM): 251 glycerol, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂·6H₂O, 2 CaCl₂, 25 NaHCO₃, 1 L-ascorbate, and 11 glucose, and oxygenated with 95% O₂/5% CO₂ (carbogen). Coronal slices (200–250 μm thick) containing the LHb were cut with a Compressed VF-200 slicer (Precisionary Instruments Inc., Greenville, NC, USA), then immediately transferred to a holding chamber and incubated for at least 1 hour at room temperature (24°–25°C) in carbogenated (95% O₂/5% CO₂) regular aCSF which has almost the same composition as glycerol-based aCSF, the exception being that the 252 mM glycerol was replaced with 126 mM NaCl. In a low Ca²⁺ solution, CaCl₂ was replaced by an equivalent amount of MgCl₂. For electrophysiological recording, a single slice was transferred to a submersion-type recording chamber and was stabilized with a platinum ring. Only one neuron was recorded from each slice.

2.2. Electrophysiology

Electrophysiological recordings were obtained with an Axon 200B amplifier, a Digidata 1440A A/D converter, and Clampfit 10.3 software (Molecular Devices Co., Union City, CA, USA). Data was filtered at 2 kHz and sampled at 5 kHz with the Digidata 1440A interface. The experiments were done at 32.6°C and maintained by an automatic temperature controller (Warner Instruments, Hamden, CT, USA). ACSF was perfused at a rate of 1.5–2 ml/min. Patch pipettes had a resistance of 6–8 MΩ when filled with an internal solution containing (in mM) 140 cesium methanesulfonate, 5 KCl, 2 MgCl₂, 10 Hepes, 2 MgATP, 0.2 GTP for voltage clamp recordings or 140 potassium gluconate, 5 KCl, 2 MgCl₂, 10 Hepes, 2 MgATP, and 0.2 GTP for current clamp recordings. The pH of these pipette solutions were adjusted to 7.2 with Tris-base and the osmolality to 310 mOsmol/L with sucrose. Excitatory postsynaptic currents (EPSCs), mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, including both evoked and spontaneous, were recorded at a holding potential (V_H) of –70 mV in the presence of gabazine (10 μM) and strychnine (1 μM) to block GABA and glycine receptors. To elicit EPSCs, a nichrome wire bipolar electrode was positioned within 200 μm of the soma, by electrical stimuli (100–200 μs in duration) at the rate of 0.05 Hz. Near the start of the recording, an input/output curve was obtained and the stimulation was then set to 20–30% of the maximum, an intensity that resulted in stable responses with no failures. Paired eEPSCs were elicited with a pair of identical stimuli separated by an interval of 50 ms. Inhibitory postsynaptic currents (IPSCs), mediated by GABA receptors were recorded at a holding potential (V_H) of +40 mV in the presence of AP5 (50 μM) and DNQX (20 μM) to block glutamate receptors. Spontaneous discharges of LHb neurons were recorded by the loose-patch cell-attached technique, which permits long-lasting recordings without perturbing the cytoplasmic contents. In some experiments, firing was recorded in whole-cell mode, which allowed for the measurement of the resting membrane potential, input resistance, and synaptic currents.

2.3. Chemicals

We purchased cocaine, cadmium chloride (CdCl₂), 6, 7-dinitroquinoxaline-2, 3-dione (DNQX), DL-2-amino-5-phosphono-valeric acid (DL-AP5), strychnine, SKF38393, SKF83566, raclopride, quinpirole, dopamine, GBR12953, gabazine, and tetrodotoxin (TTX) from Sigma (St Louis, MO). Drugs at final concentration were added to the superfusate.

2.4. Data analysis and statistics

The baseline mean values of electrophysiological data were obtained during the initial control period (3–5 min) and after washout. To assess the effects of a drug, data over a 3 min period at the peak of a drug response from each cell were obtained and then averaged. All

data were expressed as mean $\pm$ S.E.M. Statistical significance was assessed using a two-tailed Student's *t* test, a oneway ANOVA with a Tukey's *post hoc* test for multiple group comparisons, or a Kolmogorov–Smirnov test. Dose-response data were fitted to the logistic equation: $y = 100x^a / (x^a + x_0^a)$, where *y* is the percentage change, *x* is the concentration of cocaine, *a* the slope parameter, and *x*₀ the cocaine concentration which induces a half-maximal change (e.g., Fig. 2F). Significance for all analyses was determined at $p < 0.05$.

3. Results

Electrophysiological data were obtained from 200 neurons, located in both the lateral and medial sub-regions of the LHb, but mostly from the former. No significant difference was observed between data from these two sub-regions and between data from juvenile male and female rats, data were pooled.

3.1. Cocaine activates LHb neurons involving glutamate transmission

We first examined the effects of cocaine on the spontaneous firings of LHb neurons. Under current clamp, 10 μ M cocaine reversibly depolarized the cells (Fig. 1A and B), by an average of 4.6 ± 1.0 mV ($n = 6$, $t = 4.6$, $p < 0.01$, paired *t* test; Fig. 1B). Meanwhile, cocaine robustly accelerated the ongoing discharges. A similar acceleration was observed in the cell-attached mode (Fig. 1C, E and D). Data obtained from current clamp and cell-attached configurations were not significantly different and therefore were pooled. The initial spontaneous firing rate was stable in aCSF. Bath application of 10 μ M cocaine increased the firing rate by $72.3 \pm 14.9\%$, from 6.0 ± 0.8 Hz in control (aCSF) to 10.1 ± 1.1 Hz (cocaine) ($n = 17$, $t = -4.8$, $p < 0.001$; Fig. 1F). The effects of cocaine were reversed after wash. Repeated cocaine application to the same neuron produced a similar increase (Fig. 1C).

Glutamate is a major excitatory neurotransmitter, playing a crucial role in the neuronal function of many brain areas and in cocaine addiction (Cornish and Kalivas, 2000; Mameli et al., 2011; Uys and LaLumiere, 2008). The LHb receives a lot of glutamatergic inputs, including those from the lateral hypothalamus, lateral preoptic area and basal ganglia (Kowski et al., 2008; Matsumoto and Hikosaka, 2007), and the LHb neurons are largely glutamatergic (Omelchenko et al., 2009). To determine whether glutamate transmission plays a role in cocaine-induced acceleration of LHb firing, we compared the effect of cocaine in the absence and presence of the NMDA and AMPA/KA-type glutamate antagonists. The application of AP5 (50 μ M) and DNQX (20 μ M) substantially lower the spontaneous firing rate (by $43.6 \pm 13.4\%$, $n = 6$, $t = 3.3$, $p < 0.01$, paired *t* test; Fig. 1D and E), indicating that the LHb neurons were under tonic activation of glutamate transmission. When the firing reached a new stable level, the application of cocaine (10 μ M) slightly, but significantly, increased firing rate by $19.5 \pm 5.5\%$ ($n = 9$, $t = -3.5$, $p < 0.01$ vs. AP5 plus DNQX, paired *t* test; Fig. 1D, E and F). This is much less than the increase (72.3%) produced by 10 μ M cocaine in the absence of AP5 and DNQX ($t = 2.5$, $p < 0.05$, unpaired *t* test, Fig. 1F).

3.2. Cocaine enhances EPSCs in LHb neurons elicited by electrical stimulation

We next characterized the effect of cocaine on glutamate transmission. At a *V*_H of -70 mV, a membrane potential at which NMDARs do not contribute to EPSCs, we applied pairs of stimuli (50 ms interval) to evoke paired EPSCs by a local stimulating electrode. These EPSCs were completely blocked by 20 μ M DNQX (Fig. 2Aa, black dot line), indicating that they were mediated by AMPA receptors. Cocaine (10 μ M) reversibly enhanced the amplitude of the first EPSCs of each pair, by $29.6 \pm 6.6\%$ (from 28.3 ± 8.5 to 38.4 ± 13 pA, $n = 6$, $t = -4.5$, $p < 0.01$, paired *t* test), but not the second EPSCs, thus reducing the paired-pulse ratio (PPR = EPSC₂/EPSC₁) by $16.5 \pm 3.8\%$ (from 0.98 ± 0.12 before to 0.81 ± 0.1

after cocaine, $n = 6$, $t = 4.4$, $p < 0.01$, Fig. 2Aa and Ab). Since a change in the PPR usually indicates a change in presynaptic transmitter release, these data suggest that cocaine increases presynaptic glutamate release onto LHB neurons. This hypothesis is further supported by the results presented in the next section.

3.3. Cocaine facilitates the frequency but not the amplitude of spontaneous EPSCs (sEPSCs) in LHB neurons

We next examined the effect of cocaine on spontaneous EPSCs (sEPSCs), which were seen in all neurons examined in the presence of GABA and glycine antagonists at a V_H of -70 mV. Under these conditions, all sEPSCs were abolished by $20 \mu\text{M}$ DNQX (Fig. 2Ba and Bb), indicating that they were mediated by AMPA receptors. Applications of cocaine induced a robust but reversible increase in sEPSC frequency in the majority (38/42) of the LHB cells tested (Fig. 2C and D); this is further shown in Fig. 2Ea, by the sharp increase in the probability of shorter intervals between successive sEPSCs (K-S = 31%, $p < 0.001$). Cocaine's action was concentration dependent ($F_{5,46} = 6.1$, $p < 0.001$; Fig. 2F) which was well fitted by a logistic equation ($r^2 = 0.99$), giving an EC_{50} of $4 \mu\text{M}$ and a near-maximal effect of $98.1 \pm 10.0\%$ with $50 \mu\text{M}$ cocaine ($n = 6$, $p < 0.001$, Tukey's posttest).

Conversely, cocaine (0.2 – $250 \mu\text{M}$) did not significantly alter sEPSC amplitudes, as shown by the histogram in Fig. 2G. A K-S test of individual cells showed excellent agreement between control and cocaine values of sEPSC amplitudes (Fig. 2Eb). Together, these results are consistent with a presynaptic mechanism of cocaine action: enhancing excitatory transmission by increasing glutamate release.

3.4. Cocaine facilitation of sEPSCs involves calcium but not sodium channels

Both voltage-gated calcium channels and extracellular calcium play a key role in the regulation of neurotransmitter release. To determine whether cocaine's effect on EPSCs is calcium dependent, we pretreated neurons with $100 \mu\text{M}$ cadmium chloride (CdCl_2 , a non-selective blocker of voltage-gated calcium channels) or calcium free aCSF (CaCl_2 was replaced by MgCl_2) for 5 min, which substantially reduced the frequency and amplitude of sEPSCs. From the newly established baseline (in CdCl_2 or calcium free aCSF), application of cocaine ($50 \mu\text{M}$) induced a slight but significant increase in sEPSC frequency, by $15.6 \pm 5\%$ ($n = 5$, $p < 0.05$) and $33.5 \pm 7.1\%$ ($n = 6$, $p < 0.001$), respectively, without significantly changing the amplitude ($p > 0.05$, Fig. 3Ab and Ac). The increase in frequency is significantly less than cocaine's effect in normal aCSF (CdCl_2 : $t = 6.9$, $p < 0.001$; calcium free aCSF: $t = 5.3$, $p < 0.001$ vs. cocaine alone; unpaired t test).

To further characterize cocaine's effects on sEPSCs, we recorded miniature EPSCs (mEPSCs) in the presence of $0.5 \mu\text{M}$ TTX. TTX substantially lowered the frequency, by $42.6 \pm 6.1\%$ ($n = 6$, $p < 0.001$, paired t test), and the amplitude of spontaneous EPSCs, by $14.2 \pm 3.6\%$ ($n = 6$, $p < 0.01$). In the presence of TTX, cocaine ($50 \mu\text{M}$) substantially increased mEPSC frequency, by $84.3 \pm 32.9\%$ ($n = 6$, $p < 0.05$ vs. TTX; Fig. 3Aa and B), which is very similar to the increase in the absence of TTX ($t = 0.4$, $p = 0.7$, unpaired t test).

3.5. Dopamine receptors (DARs) mediate cocaine's actions on firing and glutamate transmission in LHB neurons

As illustrated in figure 1C, repeated application of cocaine to the same neuron produced a similar increase in the firing rate. This property was used to study the possible receptors involved in the actions of cocaine. We first examined, in the presence of the GABA_A antagonist gabazine ($10 \mu\text{M}$), the effect of SKF83566 ($1 \mu\text{M}$) and raclopride ($10 \mu\text{M}$), respectively the selective D1R and D2R antagonist on cocaine-induced facilitation of LHB neuron firing. While these antagonists, applied either separately or in combination did not

significantly alter the basal firing rate (Fig. 4C, left panel), they did substantially attenuated cocaine-induced facilitation of firing: 1 μM SKF83566, to $44.8 \pm 10.7\%$ of cocaine alone ($n = 6$, $t = 5.2$, $p < 0.001$, paired t test; Fig. 4B and D, left panel), 10 μM raclopride, to $56.3 \pm 12.6\%$ of cocaine alone ($n = 6$, $t = 3.5$, $p < 0.01$), 1 μM SKF83566 plus 10 μM raclopride, to $30.8 \pm 7.8\%$ of cocaine alone ($n = 6$, $t = 8.9$, $p < 0.001$; Fig. 4C, D left panel).

We next compared cocaine (10 μM)-induced facilitation of mEPSC frequency in the absence and presence of SKF83566 (1 μM) or raclopride (10 μM). Whereas these antagonists did not significantly alter mEPSC frequency (Fig. 4C, right panel), they did substantially reduce cocaine-induced facilitation to $21.6 \pm 12.6\%$ ($n = 6$, $t = 6.2$, $p < 0.001$, paired t test; in SKF83566, to $25.9 \pm 8.9\%$ ($n = 5$, $t = 8.3$, $p < 0.001$) in raclopride, and in SKF83566 plus raclopride, to $20.6 \pm 5.2\%$ ($n = 9$, $t = 15.2$, $p < 0.001$) of those produced by cocaine alone (Fig. 4B and D, right panel).

3.6. DAR agonists facilitate firing and mEPSCs of LHb neurons

We then investigated the effects of dopamine, the selective D1R (SKF38393) and D2R (quinpirole) agonists, on LHb neurons. Each of these agents (all at 10 μM) robustly accelerated spontaneous firings of LHb neurons (dopamine, by $41 \pm 7.8\%$, $n = 4$, $t = -5.3$, $p < 0.01$; SKF38393, by $36.4 \pm 8.9\%$, $n = 7$, $t = -4.1$, $p < 0.01$; and quinpirole, by $30.3 \pm 10.5\%$, $n = 6$, $t = -2.9$, $p < 0.05$, paired t test; Fig. 5A). These agents also substantially facilitated mEPSC frequency: dopamine, by $66.4 \pm 14.8\%$ ($n = 10$, $t = -4.5$, $p < 0.001$), SKF38393, by $74 \pm 18.8\%$ ($n = 8$, $t = -3.9$, $p < 0.001$) and quinpirole, by $38.4 \pm 11.5\%$ ($n = 8$, $t = -3.3$, $p < 0.01$, Fig. 5B), without significantly altering mEPSC amplitudes (data not shown).

3.7. Dopamine reuptake blocker similarly stimulates neurons in the LHb

Next we examined the effect of the specific dopamine reuptake inhibitor GBR12953. Application of GBR12953 (20 nM) significantly facilitated firing (by $15.5 \pm 4.9\%$, $n = 5$, $t = -3.2$, $p < 0.05$, Fig. 5Ca and Cc, left panel) and mEPSC frequency (by $68.6 \pm 15.3\%$, $n = 7$, $t = -4.5$, $p < 0.001$, Fig. 5Cb and Cc, right panel) without altering their amplitude (data not shown).

3.8. Effects of cocaine on GABAergic transmission in the LHb

We next tested the effect of cocaine on spontaneous inhibitory postsynaptic currents (sIPSCs). At a V_H of +40 mV and in the presence of glutamate antagonists, application of 10 μM cocaine substantially increased the sIPSC frequency in 13 of 21 LHb neurons tested (by $62.7 \pm 13.3\%$, $n = 13$, $t = -4.7$, $p < 0.001$), but significantly decreased sIPSC frequency in 8/21 neurons (by $-25.8 \pm 5.4\%$, $n = 8$, $t = 4.8$, $p < 0.01$), without significantly alter the amplitude of sIPSCs in both groups (data not show).

3.9. Cocaine induces a current in LHb neurons

To determine whether postsynaptic DARs on LHb neurons play a role in the effect of cocaine, we examined the effect of cocaine on LHb neurons clamped at a V_H of -70 mV and in the presence of a mixture of TTX (0.5 μM), strychnine (1 μM), gabazine (10 μM), AP5 (50 μM) and DNQX (20 μM), which block respectively sodium channels, glycine receptors, GABA_A receptors, NMDA- and AMPA-type glutamate receptors. Under these experimental conditions, bath perfusion of cocaine induced a slight but significant inward current (Fig. 1G) in the majority of cells tested (32/37). The cocaine-induced inward current persisted over the course of the cocaine perfusion and returned to its base line level after washout. The current amplitude depended on the concentration of cocaine ($F_{4, 27} = 13.5$, $p < 0.001$). The

maximal current induced by 50 μM cocaine was -5.5 ± 0.7 pA ($n = 5$, $p < 0.001$, Tukey's posttest). The concentration-response curve gave an EC_{50} of 3 μM ($r^2 = 0.99$, Fig. 1H).

Finally, to determine whether DARs are responsible for cocaine-induced current, we compared its peak amplitude in the absence and presence of DAR antagonists. The currents induced by 10 μM cocaine was abolished by 10 μM SKF83566 plus 10 μM raclopride, from 5.3 ± 1.0 pA to 0.2 ± 0.6 pA ($n = 4$, $t = 4.3$, $p < 0.05$; paired t test).

4. Discussion

We report here the electrophysiological evidence that cocaine accelerates LHb neuron firing and increases glutamate transmission to the LHb neurons. This action of cocaine may be mediated mainly by presynaptic D1Rs and D2Rs that exist on the glutamatergic terminals which make synapses on the LHb neurons. Given that glutamate transmission plays a major role in cocaine addiction and the LHb is a key player in aversion, these actions of cocaine may contribute to its aversive property.

4.1. The mechanism of cocaine-induced excitation of LHb neurons

Glutamate transmission accounts for 80 – 90% of the excitatory synaptic signaling in the mammalian CNS (Schmidt and Pierce, 2010). In this study we showed that cocaine-induced acceleration of LHb neuron firing was substantially attenuated by antagonists of glutamate receptors, indicating that these receptors play a major role. This possibility is further supported by cocaine-induced increase in presynaptic glutamate release: enhances the amplitude but decreases the paired-pulse ratio of evoked EPSCs; increased the frequency of sEPSC/mEPSCs, without altering their amplitude. Our data are consistent with a recent report showing that cocaine potentiates EPSCs in LHb neurons (Maroteaux and Mameli, 2012). Additionally, we showed that cocaine's action depended on calcium but not TTX-sensitive sodium channels, consistent with the recent report that cocaine-induced glutamate release relies on calcium (Wolf and Tseng, 2012).

The changes in the LHb neuron activity may reflect the net effects of integration of the pre-synaptic and post-synaptic actions of cocaine. It is worthily noted that cocaine-induced facilitation of LHb firings was substantially attenuated but not abolished by AP5 and DNQX, indicating that mechanisms other than AMPA and NMDA receptors may also be involved. This possibility is supported by our data showing that cocaine suppressed sIPSCs in a portion of LHb neurons tested, which may contribute partly to cocaine-induced excitation in these neurons. Interestingly, cocaine facilitated sIPSCs in a larger portion of LHb neurons. The role of the GABAergic IPSCs in cocaine-induced facilitation of LHb firing in these neurons is unclear and warrants further investigation. However, cocaine facilitated firing of LHb neurons in the presence of gabazine (Fig. 4), indicating that cocaine can excite LHb neurons independent of GABA_ARs. In addition, in the presence of antagonists which block glutamate, GABA and glycine transmissions and action potentials, cocaine induced an inward current which could contribute to membrane depolarization (Fig. 1H) and acceleration of firing. The observation that cocaine-induced current was abolished by DAR antagonists indicates that the postsynaptic DARs also contribute to cocaine-induced excitation of LHb neurons. However, since co-application of D1R and D2R antagonists did not completely abolish cocaine's effect of LHb firing, mechanisms other than DARs may also be involved. Indeed, a previous study showing that cocaine may directly excite LHb neurons by attenuating the delayed rectifier potassium current (Zhang et al., 2007). In addition, cocaine blocks also the reuptake of serotonin and noradrenalin (Ritz et al., 1990). How serotonin and noradrenalin contribute to cocaine's effects on LHb firing is currently unknown and warrants further investigation.

4.2. Dopamine signaling in the LHb

Cocaine's effect seems to rely on its ability to increase the availability of monoamines, such as dopamine, serotonin and noradrenaline (Ritz et al., 1990). Although cocaine has multiple sites of action, a major effect is to increase extracellular dopamine levels by inhibiting the DAT, and this has long been thought to mediate most of its behavioral effects, including locomotor stimulation and reward (Kuhar et al., 1991).

Our data reveal that the LHb neurons are subjected to dopamine regulation: dopamine, SKF 38393, quinpirole and GBR12953 all facilitated mEPSC frequency in LHb neurons, and cocaine's facilitation of mEPSCs was blocked by either D1R or D2R antagonists. This is in line with previous reports which show that: (i) The LHb receives dense projections from the midbrain dopaminergic neurons (Gruber et al., 2007; Phillipson and Griffith, 1980; Skagerberg et al., 1984); (ii) The LHb has a significant amount of dopamine (Kizer et al., 1976; Phillipson and Pycock, 1982; Versteeg et al., 1976); (iii) The LHb has rich DAR expression (GENSAT, 2008; Weiner et al., 1991); and (iv) The LHb activity and c-fos gene expression are substantially altered by systemic or local administration of dopamine and dopamine agonists (Kowski et al., 2009; Kozlowski and Marshall, 1980; McCulloch et al., 1982; McCulloch et al., 1980; Reisine et al., 1984; Trugman et al., 1991).

Intriguingly, quinpirole accelerates the spontaneous firings and mEPSCs of LHb neurons. Quinpirole is a selective D2-family agonist, with high affinity for D2, D3, and D4 DARs. Conversely, the D2R antagonist raclopride suppresses the excitatory effect of cocaine on both firing and mEPSCs of LHb neurons. These data indicate that activation of D2Rs increases the excitability of LHb neurons. Interestingly, D2R activation increases neuronal excitability has also been reported in several other brain areas, including the prefrontal cortex (Benoit-Marand and O'Donnell, 2008; Gee et al., 2012; Wang and Goldman-Rakic, 2004), nucleus accumbens (Huppe-Gourgues and O'Donnell, 2012), and somatosensory thalamocortex (Govindaiah et al., 2010). Although the underlying mechanism is unclear, it has been postulated that this excitatory effect of D2R is independent of Gi, but may involve activation of calcium channels (Gee et al., 2012) or the Na⁺/Ca²⁺ exchanger (Yanovsky et al., 2011).

4.3. Functional implication

In addition to its rewarding property, cocaine is known to have strong aversive effects. The LHb is one of the few regions in the brain to be excited by aversive stimuli or the absence of expected reward (Bromberg-Martin and Hikosaka, 2011). Previous studies have suggested that the LHb contributes significantly to cocaine-induced aversive effect. For example, when the LHb is lesion or inactivated, cocaine intake is enhanced (Friedman et al., 2010). The glutamatergic LHb neurons are mainly projecting to the GABAergic RMTg neurons. Activation of the LHb neurons will increase glutamate release in the RMTg and activate the RMTg neurons, which release GABA onto dopamine cells in the midbrain. Thus, LHb neuron activation will suppress dopamine neurons and may contribute to the aversive effect. The data presented in this study suggest that cocaine, probably by blocking the DATs, could increase LHb dopamine levels, which in turn activate the D1Rs and D2Rs on the glutamatergic terminals synapsing on the LHb neurons and activate these neurons. The activation of LHb neurons may contribute to the aversive effect of cocaine.

5. Conclusion

Our results show that the LHb is a major target of cocaine. Although the underlying mechanisms of how cocaine activates LHb neurons have yet to be completely elucidated, our data suggest that these effects are mediated at least in part by D1Rs and D2Rs located presynaptically on glutamatergic afferents innervating LHb neurons, as well as on the

somatodendritic regions of these neurons. These effects of cocaine may contribute to the aversive properties of cocaine.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AP5	DL-2-amino-5-phosphono-valeric acid
DA	dopamine
DNQX	6, 7-dinitroquinoxaline-2, 3-dione
Hb	Habenula complex
LHb	Lateral habenula
sEPSCs	spontaneous excitatory postsynaptic currents
mEPSCs	miniature excitatory postsynaptic current
TTX	tetrodotoxin

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- Cocaine depolarizes LHb neurons and accelerates their ongoing firing.
- Cocaine enhances glutamate excitatory transmission.
- Dopamine, D1, D2 agonists, dopamine reuptake blocker mimicked cocaine's effect.
- Both D1 and D2 antagonists blocked cocaine's effects on EPSCs.

Fig. 1.

Cocaine depolarizes LHB neurons and accelerates their firing. **(A)** Exemplar traces of cocaine-induced changes in membrane potential and spontaneous firings. Dashed-lines indicate resting membrane potentials in control and in cocaine. **(B)** Histogram is mean ($\pm$ SEM) of membrane depolarization induced by 10 μ M cocaine. Number of cells is indicated. Panels **C–E**: Cocaine-induced increase in firing of LHB neurons recorded in cell-attached mode. For all figures, the bar above the voltage (or current) trace signals the application of the indicated drug. **(C)** Repeated applications of 10 μ M cocaine produced similar increases in firing rate. **(D)** Current traces at times indicated in **E**. **(E)** Time-course of cocaine-induced excitation on an LHB neuron in the absence and presence of glutamate receptors antagonists (50 μ M AP5 plus 20 μ M DNQX). **(F)** Cocaine (10 μ M) increases the firing rate of LHB neurons in the absence and presence of (50 μ M AP5 plus 20 μ M DNQX. * p < 0.05, ** p < 0.01, *** p < 0.001, Student's paired t test for cocaine application vs. pre-cocaine control. # p < 0.05, unpaired t test for cocaine alone vs. cocaine plus glutamate receptor antagonists. **(G)** Exemplar current trace induced by 10 μ M cocaine in an LHB neuron at a V_H of -70 mV in the presence of 0.5 μ M TTX, 50 μ M AP5, 20 μ M DNQX, 10 μ M gabazine, and 1 μ M

strychnine. Red dotted line indicates baseline current extrapolated from the recording prior to cocaine application. (**H**) Concentration-response relationship of cocaine-induced inward currents. ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA followed by Tukey's multiple comparisons test. Cell numbers are indicated.

Fig. 2.

Cocaine enhances the amplitude of evoked EPSCs and the frequency of spontaneous EPSCs in LHB neurons. (**Aa**) Exemplar traces of EPSCs from a single cell, during paired-pulse stimulation, cocaine reversibly increased the amplitude of the first but not second EPSCs. The trace was abolished by DNQX, indicating that it was mediated by AMPA-type glutamate receptors. (**Ab**) Summary of % change in eEPSC amplitude and paired-pulse ratio (PPR = EPSC₂/EPSC₁). **p < 0.01, Student's paired *t* test for cocaine application vs. pre-cocaine control. Recorded at a V_H of -70 mV and in the presence of gabazine and strychnine, these sEPSCs (**Ba**) were completely abolished by AMPA-type glutamate antagonist DNQX (**Bb**). (**C**) Examples of sEPSCs obtained at times indicated in **D**. (**D**) Time course of cocaine induced changes in sEPSC frequency. Cumulative probability plots show cocaine increased incidence of shorter intervals (**Ea**, K-S test, $p < 0.001$, cocaine vs. control), but did not alter the amplitude (**Eb**, K-S test, $p = 0.51$) between sEPSCs from the same neuron shown in **D**. Concentration-response relationship of cocaine-induced facilitation (% increase) of sEPSC frequency (**F**). The smooth curve is the best fit of the data to the logistic equation. Number of cells is indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA followed by Tukey's multiple comparisons test. (**G**) Cocaine (0.2–250 μ M) did not significantly alter sEPSC amplitude.

Fig. 3.

Cocaine enhances frequency of miniature EPSCs. **(A)** Time course of cocaine-induced increase in sEPSC frequency in the presence of **a**, TTX (sodium channel blocker), **b**, CdCl₂ (calcium channel blocker), and **c**, calcium free aCSF. **(B)** Relative increases in sEPSC frequency induced by 10 μ M cocaine in the absence (control) and presence of TTX, CdCl₂ or calcium free aCSF. Number of neurons is indicated. * $p < 0.05$, *** $p < 0.001$, Student's paired t test for cocaine vs. pre-cocaine control. ### $p < 0.001$, Student's unpaired t test for cocaine vs. cocaine plus TTX/ CdCl₂/ calcium free aCSF.

Fig. 4.

Both D1R antagonist SKF83566 and D2R antagonist raclopride depress cocaine-induced acceleration of firing and mEPSCs. Time courses of cocaine effect on the frequency of firing (**A**) and mEPSCs (**B**) in the absence (aCSF+10 μ M gabazine) and presence of 10 μ M SKF83566 (**a**), 10 μ M raclopride (**b**) or 10 μ M SKF83566 plus 10 μ M raclopride (**c**). (**C**) Summary (Mean $\pm$ S.E.M.) of SKF83566, or raclopride, or SKF83566 plus raclopride induced change (%) of firing and mEPSC frequency. (**D**) Summary of the results from A and B, normalized to the increase induced by 10 μ M cocaine alone. ** p < 0.01, *** p < 0.001, Student's paired t test for cocaine vs. cocaine plus SKF83566/ raclopride/ SKF83566 plus raclopride.

Fig. 5.

Dopamine (DA), D1R agonist, D2R agonist or dopamine transport blocker mimic the effect of cocaine on firing and mEPSC frequency in LHb neurons induced respectively by **a**, dopamine, **b**, D1R agonist SKF38393, and **c**, D2R agonist quinpirole. (**Ad**, **Bd**) Summary (Mean $\pm$ S.E.M.) of facilitation (% increase) of firing and mEPSC frequency induced by dopamine, SKF38393, or quinpirole (**C**) Time courses of increases in firing (**a**) and mEPSC frequency (**b**) induced by GBR12953 (20 nM, a dopamine transport blocker), cocaine produced similar facilitation in the same neurons. (**Cc**) Summary (Mean $\pm$ S.E.M.) of facilitation (% increase) of firing and mEPSC frequency induced by 20 nM GBR12953. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's paired t test for drug application vs. pre-drug control.