

# Ventral Subiculum Stimulation Promotes Persistent Hyperactivity of Dopamine Neurons and Facilitates Behavioral Effects of Cocaine

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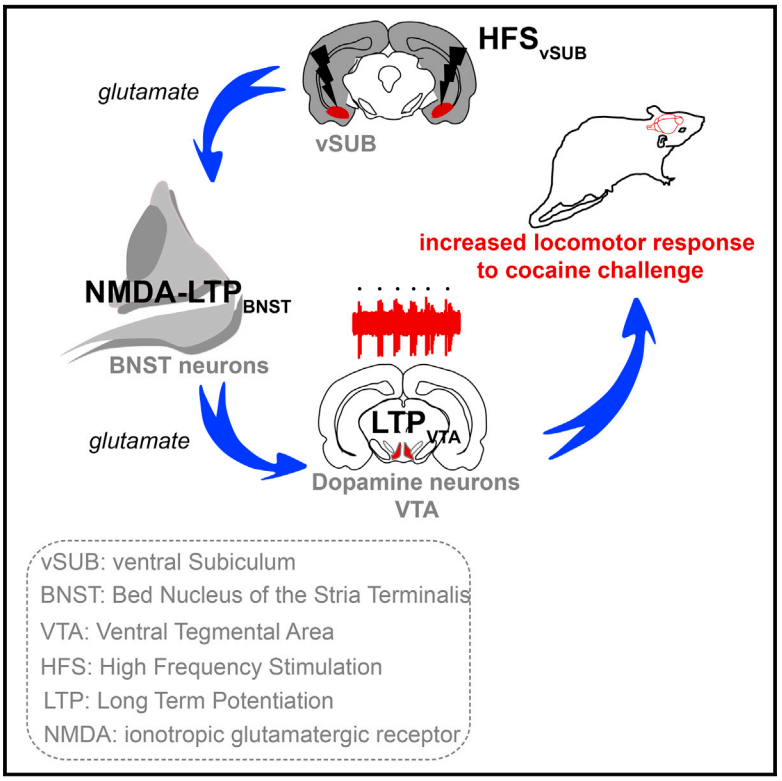
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# Ventral Subiculum Stimulation Promotes Persistent Hyperactivity of Dopamine Neurons and Facilitates Behavioral Effects of Cocaine

## Graphical Abstract




## Highlights

- vSUB controls the phasic activity of VTA dopamine neurons
- The BNST relays the excitatory drive from the vSUB to VTA dopamine neurons
- vSUB stimulation promotes persistent hyperactivity of VTA dopamine neurons
- vSUB-driven NMDA-LTP in the BNST gates cocaine-induced locomotor activity

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## In Brief

Glangetas et al. show that the ventral subiculum alters the excitability of dopamine neurons in vivo via a relay within the bed nucleus of the stria terminalis, revealing a neuronal circuit controlling behavioral effects of cocaine.

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# Ventral Subiculum Stimulation Promotes Persistent Hyperactivity of Dopamine Neurons and Facilitates Behavioral Effects of Cocaine

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## SUMMARY

**The ventral subiculum (vSUB) plays a key role in addiction, and identifying the neuronal circuits and synaptic mechanisms by which vSUB alters the excitability of dopamine neurons is a necessary step to understand the motor changes induced by cocaine. Here, we report that high-frequency stimulation of the vSUB (HFSvSUB) over-activates ventral tegmental area (VTA) dopamine neurons in vivo and triggers long-lasting modifications of synaptic transmission measured ex vivo. This potentiation is caused by NMDA-dependent plastic changes occurring in the bed nucleus of the stria terminalis (BNST). Finally, we report that the modification of the BNST-VTA neural circuits induced by HFSvSUB potentiates locomotor activity induced by a sub-threshold dose of cocaine. Our findings unravel a neuronal circuit encoding behavioral effects of cocaine in rats and highlight the importance of adaptive modifications in the BNST, a structure that influences motivated behavior as well as maladaptive behaviors associated with addiction.**

## INTRODUCTION

The circuit-based plastic adaptation of ventral tegmental area (VTA) dopamine neurons is an indispensable initial step in reward-related circuitry, leading to the behavioral effects of cocaine (Zweifel et al., 2008). Recently, it has been shown that interactions between the hippocampus and the VTA are important for context-reward associations (Luo et al., 2011). The ventral subiculum (vSUB), which is the output region of the ventral hippocampus, plays a critical role in several fundamental cognitive functions, including the integration of relevant contextual information to modulate goal-directed behaviors as well as behaviors associated with cocaine seeking (Sesack and Grace, 2010; Sun and Rebec, 2003; Vorel et al., 2001). The vSUB controls the activity of dopamine neurons (Floresco et al., 2001a; Legault et al., 2000; Legault and Wise, 1999; Todd and Grace, 1999), and it is well established that stimulation of the vSUB induces a release of dopamine in the nucleus accumbens (Nac) shell, which is associated with an increase in locomotor activity (Bardgett and Henry, 1999; Brudzynski and Gibson, 1997; Giménez-Llort et al., 2002; Peleg-Raibstein and Feldon, 2006; Yang and Mogenson, 1985). Two mechanisms have been proposed to account for the activation of dopamine neurons in response to vSUB stimulation. The first assumes that vSUB inputs to the Nac lead to the disinhibition of dopamine neuron activity and control the proportion of spontaneously active dopamine neurons (Floresco et al., 2001a). The second proposed mechanism involves a glutamatergic structure that would receive vSUB inputs and project to the VTA, thereby controlling the activation of the dopamine neurons in response to vSUB stimulation (Legault et al., 2000). This mechanism is further supported by demonstrating that (1) high-frequency electrical stimulation of the vSUB ($HFS_{vSUB}$) triggers cocaine-seeking behavior via modulation of glutamate release in the VTA (Vorel et al., 2001), (2) synaptic plasticity at excitatory synapses onto dopamine neurons is thought to represent the cellular correlate of initiating addictive behavior (Chen et al., 2010; Creed and Lüscher, 2013), and finally that (3) glutamatergic activation of

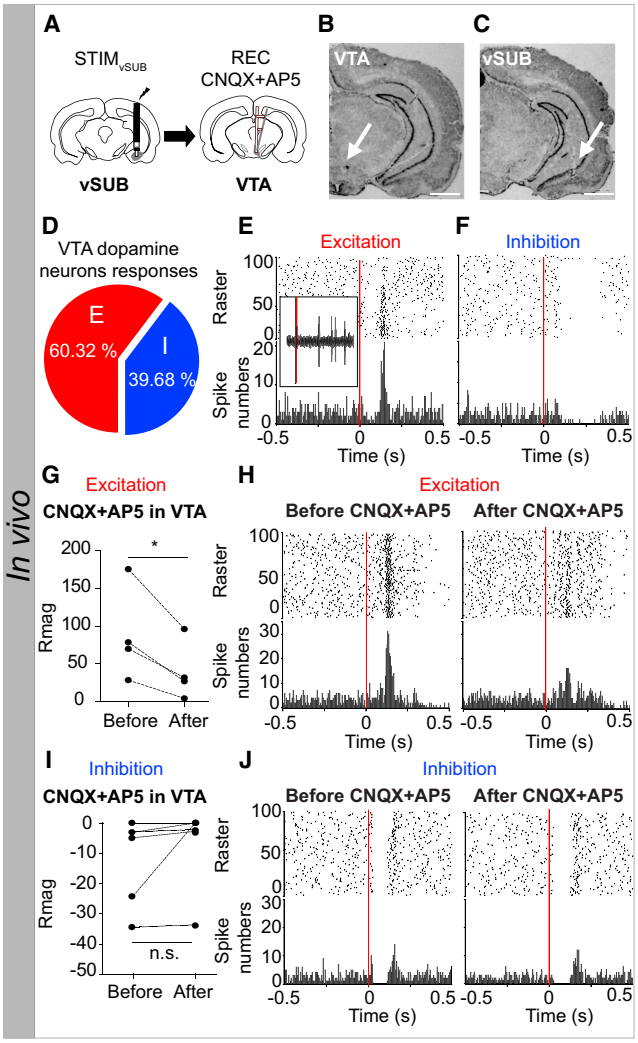



**Figure 1. The vSUB Controls the Phasic Activity of VTA Dopamine Neurons**

(A) Experimental protocol.

(B and C) Histological controls of the recording site (B; VTA, white arrow) and the stimulation area (C; vSUB, white arrow). The scale bar represents 1.0 mm.

(D) Quantification of VTA dopamine neuron responses to electrical stimulation of the vSUB.

(E and F) Typical peristimulus time histograms (PSTHs) show vSUB-evoked responses of VTA dopamine neurons; (E) excitation; (F) inhibition. Red line represents the artifact of stimulation (t0).

(G) Graph illustrating the effect of ionotropic glutamatergic receptor antagonist (CNQX+AP5) on VTA dopamine neuron excitation obtained after vSUB stimulation.

(H) Typical PSTHs show vSUB-evoked response (excitation) of a VTA dopamine neuron before and after CNQX and AP5 injection into VTA. Rmag, excitatory response magnitude.

(I) Graph illustrating the effect of ionotropic glutamatergic receptor antagonist (CNQX+AP5) on VTA dopamine neuron inhibition evoked by vSUB stimulation.

(J) Typical PSTHs showing vSUB-evoked inhibitory responses of a VTA dopamine neuron before and after CNQX+AP5 infusion into VTA.

the VTA is necessary for cocaine-evoked dopamine release in the Nac and cocaine-seeking behavior (Engblom et al., 2008; Sombers et al., 2009).

However, the glutamatergic circuit by which vSUB and VTA interact remains to be elucidated. The anteromedial area of the bed nucleus of the stria terminalis (amBNST) represents a potential candidate. It receives a dense innervation from the vSUB (Cullinan et al., 1993), and we recently reported that the amBNST is a hub channeling brain information from the vSUB toward VTA dopamine neurons (Jalabert et al., 2009). Furthermore, the amBNST controls the activity of dopamine neurons (Cullinan et al., 1993; Georges and Aston-Jones, 2001; Jennings et al., 2013; Kudo et al., 2012) and is highly involved in emotional behavior associated with cocaine seeking and aversion (de-Backer et al., 2015; Dumont et al., 2005; Jennings et al., 2013). In particular, amBNST afferents to the VTA are thought to be important for cocaine preference (Briand et al., 2010). These results led to the hypothesis that synaptic plasticity in the amBNST driven by a single episode of $HFS_{vSUB}$ could trigger hyperactivity of dopamine neurons that would in turn influence the locomotor effect of cocaine.

To investigate how VTA dopamine neurons integrate excitatory information from the vSUB and the mechanism of their adaptive changes in response to a single episode of $HFS_{vSUB}$ stimulation, we combined in vivo single-cell recordings, in vitro patch-clamp recording, tracing, and pharmacological and behavioral approaches. Here, we demonstrate that (1) electrical stimulation of the vSUB phasically drives the activity of VTA dopamine neurons, (2) the amBNST relays glutamatergic information from the vSUB to the VTA, (3) $HFS_{vSUB}$ induces an in vivo NMDA-dependent long-term potentiation (LTP) at the vSUB-amBNST synapses of amBNST neurons projecting to the VTA ($amBNST_{\rightarrow VTA}$ neurons), (4) $HFS_{vSUB}$-induced LTP in the amBNST is permissive for the hyperactivity of tonic activity of VTA dopamine neurons, and (5) $HFS_{vSUB}$ potentiates locomotor effect of cocaine in a amBNST-dependent manner.

## RESULTS

### The vSUB Controls the Phasic Activity of VTA Dopamine Neurons

In a first set of experiments, we sought to unravel the neuronal network by which the vSUB drives the activity of VTA dopamine neurons (Figure 1). First, we measured the evoked activity from single VTA dopamine neurons identified according to their electrophysiological properties (see Experimental Procedures) in response to single-pulse stimulation of the vSUB at 0.5 Hz (Figures 1A–1C). We showed that this stimulation in the vSUB controls the phasic activity of VTA dopamine neurons (Figures 1D–1F). 85.2% of the total VTA dopamine recorded neurons responded to this stimulation (n = 54 total VTA dopamine recorded neurons; n = 9 rats). Stimulation of the vSUB evoked excitatory responses in 60.32% of the VTA dopamine neurons and inhibitory responses in 39.68% of recorded neurons (Figures 1D–1F). The onset of excitation was 124.47 ± 10.35 ms, and the onset of inhibition was 67.6 ± 9.13 ms. Local infusion of glutamatergic receptor antagonists (CNQX+AP5) in the VTA drastically reduced the vSUB-evoked excitatory responses in VTA dopamine neurons (Rmag = 88.08 ± 31.08 evoked spikes before CNQX+AP5 injection; Rmag = 39.83 ± 19.69 evoked spikes after CNQX+AP5 injection; $p < 0.05$; Figures 1G

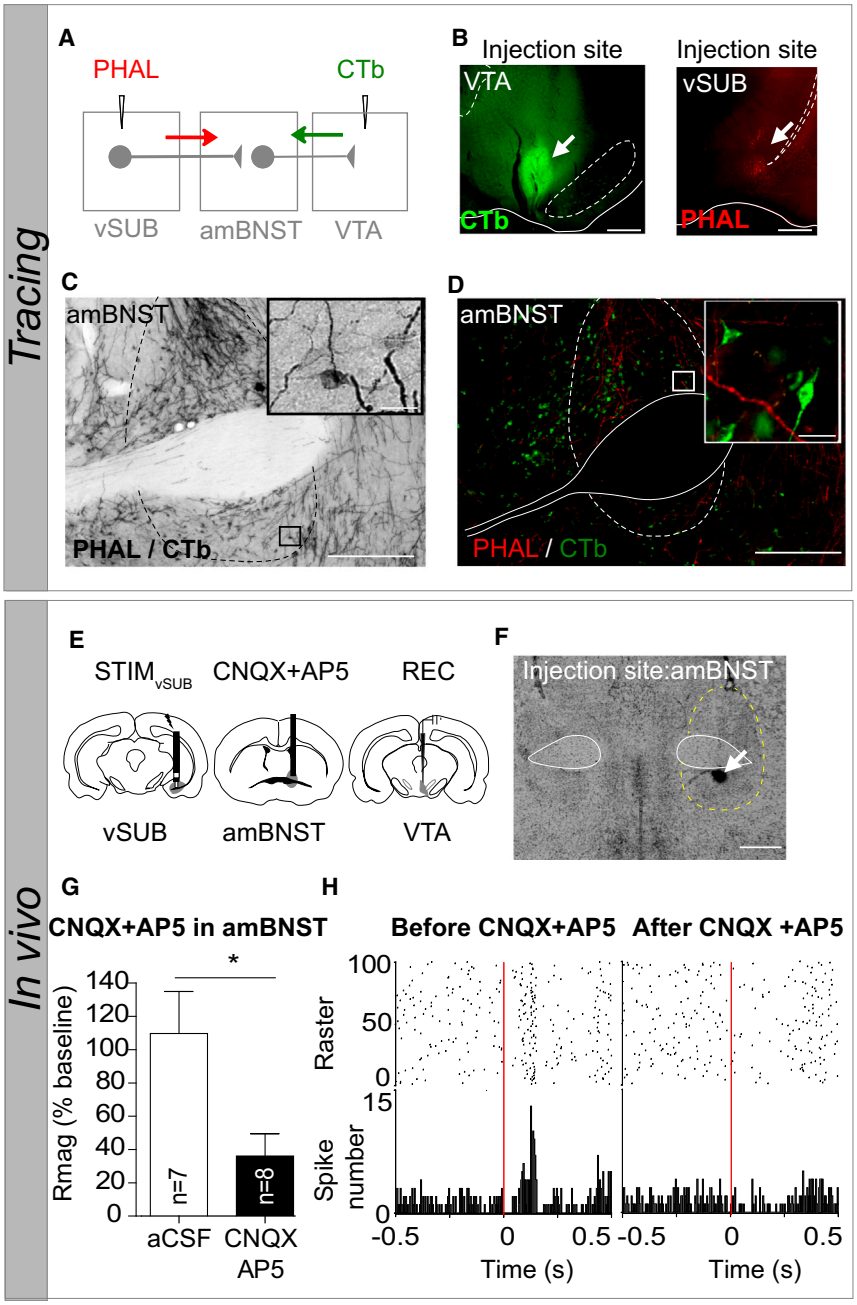



**Figure 2. The amBNST Is an Excitatory Relay from the vSUB to the VTA**

(A) Experimental protocol.

(B) Injection site of a retrograde tracer CTb in the VTA (left) and anterograde tracer PHAL in the vSUB (right). The scale bar represents 1 mm.

(C and D) Photographs showing PHAL-positive terminal fibers and CTb-positive neurons in the amBNST with DAB nickel labeling (C) or with fluorescent labeling (D). The scale bar represents 0.5 mm and scale bar inset represents 10 μm.

(E) Experimental protocol.

(F) Histological control of the injection site of CNQX and AP5 in the amBNST. The scale bar represents 0.5 mm.

(G) Graph showing the effect of ionotropic glutamatergic receptor antagonist injection (or its vehicle, aCSF) into the amBNST on VTA dopamine neuron excitation normalized to the baseline after the $STIM_{vSUB}$.

(H) Typical PSTHs show vSUB-evoked response (excitation) of a VTA dopamine neuron before and after CNQX and AP5 injection into the amBNST.

and 1H) without affecting the vSUB-evoked inhibitory responses amplitude (Rmag = −11.5 ± 5.79 evoked spikes before CNQX+AP5 injection; Rmag = −6.42 ± 5.47 evoked spikes after CNQX+AP5 injection; $p > 0.05$; Figures 1I and 1J) or onset latencies (53.1 ± 14.39 ms before CNQX+AP5 injection; Rmag = 45.8 ± 14.11 ms after CNQX+AP5 injection; $p > 0.05$). Altogether, these data demonstrated a mixed excitatory and inhibitory influence of the vSUB on the VTA dopamine neuron activity. It revealed the involvement of a glutamatergic link terminating in the VTA and controlling the excitation of the dopamine neurons in response to vSUB stimulation.

## The amBNST Relays the Excitatory Drive from the vSUB to the VTA

The amBNST is thought to be a functional glutamatergic input to the VTA that helps controlling the activity of dopamine neurons (Georges and Aston-Jones, 2002; Jennings et al., 2013; Kudo et al., 2012). By using tracing approach and pharmacological manipulations, we examined the role of amBNST as functional excitatory relay between the vSUB and the VTA (Figure 2; Jalabert et al., 2009). By injecting a retrograde tracer, the cholera toxin B subunit (CTb), into the VTA, we first confirmed that the VTA receives strong projections from the amBNST (Figures S1A–S1C). We also noted that very few neurons from the vSUB directly project to the VTA (Figures S1D and S1E). We then examined whether the amBNST acts as an anatomical and functional relay between the vSUB and the VTA (Figure 2). A double-labeling strategy combining injection of the anterograde tracer, *Phaseolus vulgaris* agglutinin (PHAL), into the vSUB and injection of CTb into the VTA confirmed the role of the amBNST as an anatomical relay between the vSUB and the VTA (Figures 2A–2D). The greatest density of CTb-labeled neurons was found in the dorsal and ventral portion of the amBNST, coincident with the PHAL-hippocampal labeling originating from the vSUB. To test whether the amBNST could be a glutamatergic link within the polysynaptic vSUB-VTA pathway, we recorded VTA dopamine neuron responses to vSUB stimulation while inactivating amBNST with CNQX+AP5 (Figures 2E and 2F). Injection of CNQX+AP5 into the amBNST decreased the VTA dopamine neuron excitatory response evoked by vSUB

stimulation (aCSF, Rmag = 109.8% ± 25.21% of the baseline; CNQX+AP5, Rmag = 36.1% ± 13.37% of the baseline; $p < 0.05$; Figures 2G and 2H). This experiment indicates that the amBNST acts as a relay integrating excitatory inputs from vSUB and in turn controlling the VTA dopamine neurons.

## Stimulation of vSUB Elicits In Vivo Persistent Hyperactivity of VTA Dopamine Neurons

Next, we addressed the question of the long-term effect of a single episode of high-frequency stimulation of the ventral subiculum ($HFS_{vSUB}$) on the tonic activity of VTA dopamine neurons. To this end, we used a physiologically relevant stimulation protocol (HFS) in anesthetized rats that has been shown to trigger a relapse to cocaine seeking (Vorel et al., 2001). In a first set of experiments, we investigated the long-term effect of $HFS_{vSUB}$ on the tonic activity of VTA dopamine neurons. Given the control of vSUB over the VTA through the amBNST, we predicted that $HFS_{vSUB}$ might engage long-term modifications in activity and synaptic strength in the VTA. The impact of $HFS_{vSUB}$ on VTA dopamine neurons was measured at two time points, i.e., after 1 and 5 days (Figures 3A–3H). The firing rate of VTA dopamine neurons increased 5 days after $HFS_{vSUB}$ compared to the SHAM group (firing rate: SHAM group: 2.99 ± 0.2 Hz; 5D-$HFS_{vSUB}$ group: 4.55 ± 0.33 Hz; $F_{(2,220)} = 9.183$; $p < 0.0001$; Figure 3E). The bursting parameters of VTA dopamine neurons increased after $HFS_{vSUB}$ compared to the SHAM group (Figures 3F–3H). The bursting rate and the percentage of spikes in burst increased 1 day after $HFS_{vSUB}$ compared to the SHAM group and were even higher 5 days after $HFS_{vSUB}$ (bursting rate: SHAM group: 0.27 ± 0.04 Hz; 1D-$HFS_{vSUB}$: 0.41 ± 0.04 Hz; 5D-$HFS_{vSUB}$: 0.66 ± 0.08 Hz; $F_{(2,220)} = 11.55$; $p < 0.0001$; Figure 3F; spike in bursts: SHAM group 21.9% ± 2.83%; 1D $HFS_{vSUB}$ 32.52% ± 2.58%; 5D $HFS_{vSUB}$ 44.15% ± 4.8%; $F_{(2,220)} = 8.915$; $p < 0.0002$; Figure 3G; mean spike per burst $F_{(2,220)} = 5.958$; $p < 0.01$; Figure 3H). Overall, $HFS_{vSUB}$ elicits in vivo persistent hyperactivity of VTA dopamine neurons.

Such increased activity could be due to an activity-dependent increased synaptic efficacy in dopamine neurons, a phenomenon that has been implicated in various forms of motivated behaviors (Stuber et al., 2008; Ungless et al., 2001). To test whether $HFS_{vSUB}$ would produce long-lasting synaptic modifications in DA neurons, we used whole-cell patch recordings in acute slices. We measured AMPA- and NMDA-mediated excitatory postsynaptic currents (EPSCs) in voltage clamp mode at +40 mV in slices obtained from SHAM and 5D-$HFS_{vSUB}$ rats. In 5D-$HFS_{vSUB}$ rats, the AMPA/NMDA ratio increased more than in the SHAM rats (SHAM group 0.57 ± 0.1, n = 13, n = 5 rats; 5D-$HFS_{vSUB}$ group: 1.14 ± 0.2, n = 11 neurons, n = 5 rats; $p < 0.05$; Figure S2). Altogether, these results indicate that $HFS_{vSUB}$ promotes both an increase in their firing and bursting in vivo properties and long-lasting modifications of synaptic transmission to VTA dopamine neurons measured ex vivo.

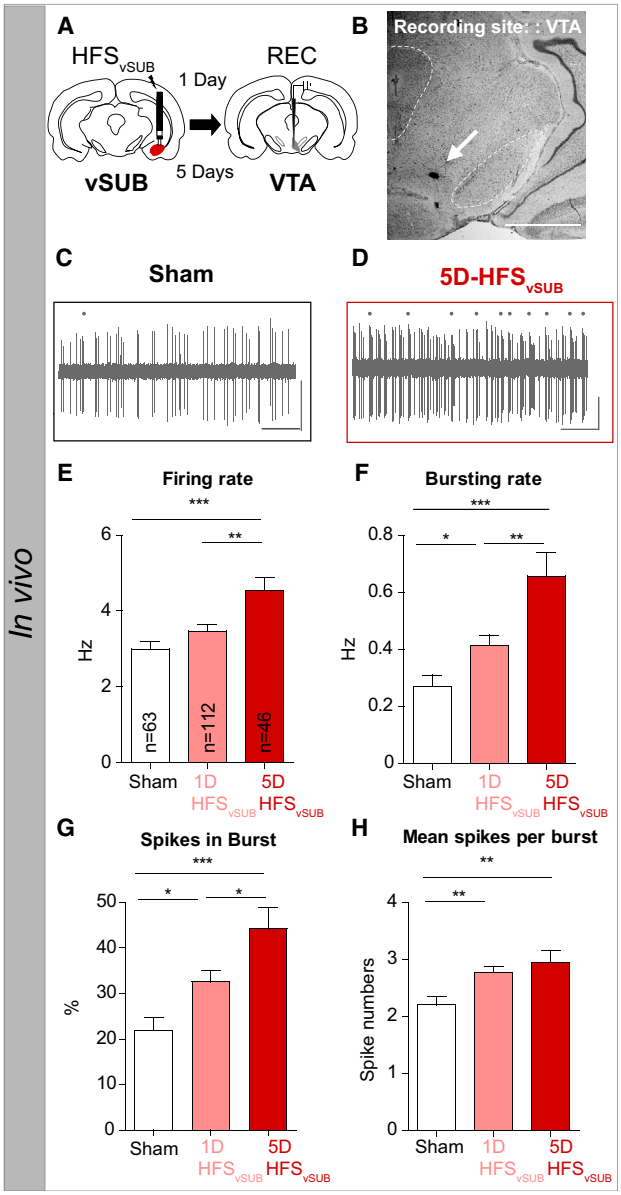



**Figure 3. In Vivo Potentiation of VTA Dopamine Neurons Induced by $HFS_{vSUB}$**

(A) Experimental protocol.

(B) Histological control of the recording site: the VTA. The scale bar represents 1.0 mm.

(C and D) Representative trace of a VTA dopamine neuron recorded 5 days after $HFS_{vSUB}$ (5D-$HFS_{vSUB}$) or without any stimulation (SHAM). Each dot represents a burst event. The scale bar represents 2.5 ms; 0.5 V.

(E–H) Graphs illustrating the long-term effect of $HFS_{vSUB}$ on VTA dopamine neuron tonic firing activity (E) and bursting parameters (F–H) 1 day or 5 days after the $HFS_{vSUB}$. $HFS_{vSUB}$, high-frequency stimulation of the vSUB; REC, recording; 1D, 1 day; 5D, 5 days.

## vSUB Stimulation Hyperactivates VTA Dopamine Neurons through the BNST-VTA Relay

How such hyperactivation is achieved remains unclear, but our anatomical results suggest the involvement of the BNST-VTA pathway. We thus investigated the impact of $HFS_{vSUB}$ on the excitability properties of amBNST neurons projecting to the VTA ($amBNST_{\rightarrow VTA}$ neurons) in response to electrical stimulation of the vSUB (Figure 4). Antidromic activation of

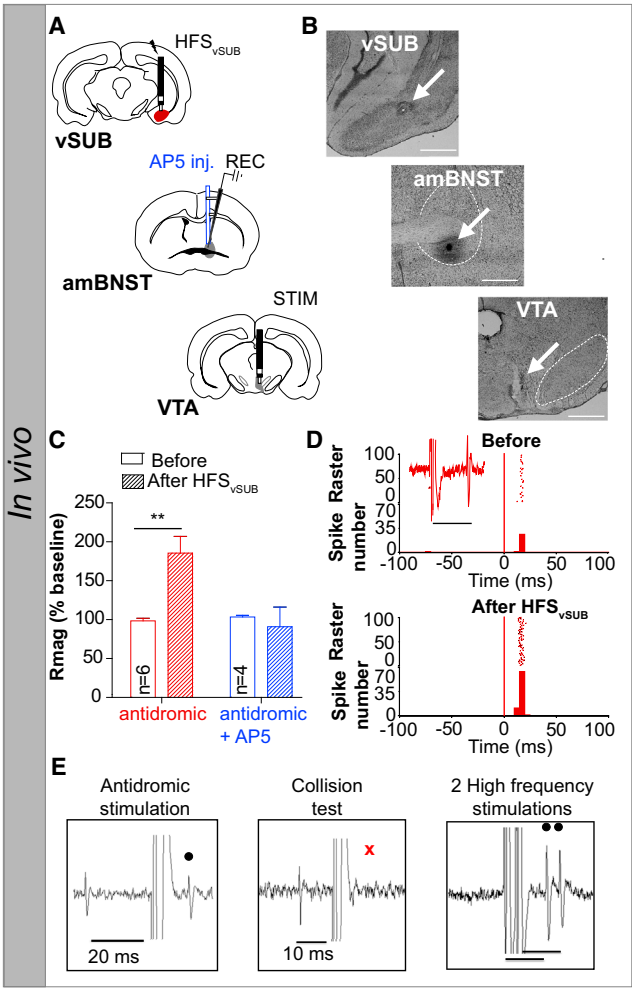



**Figure 4. $HFS_{vSUB}$ Induces LTP in VTA-Projecting amBNST Neurons**

(A) Experimental protocol.

(B) Histological control of the recording site, the BNST, and the stimulating sites, the vSUB and the VTA (white arrow). The scale bar represents 1.0 mm.

(C) Quantification of the mean percentage change (±SEM) in the vSUB-evoked spike probability in amBNST neurons projecting to the VTA normalized to the baseline after $HFS_{vSUB}$ in rats.

(D) Typical PSTH shows vSUB-evoked response (excitation) of a BNST neuron projecting to the VTA before and after $HFS_{vSUB}$.

(E) Representative traces showing a collision test and a high-frequency stimulation protocol for an amBNST neuron driven from the VTA. Left: Stimulation of the VTA 20 ms after spontaneous spikes elicits a driven spike (black circle) at 9.5-ms latency is shown. Middle: Driven spikes are occluded for similar stimuli delivered 10 ms after spontaneous impulses, indicating collision between spontaneous and driven spikes. The red cross indicates when driven spikes would have occurred in the absence of collision. Right: Driven antidromic spikes (black circles) elicited by each of the paired stimuli (2-ms interpulse interval) demonstrate that the cell can follow a frequency of 500 Hz.

amBNST neurons following VTA stimulation was used to identify $amBNST_{\rightarrow VTA}$ neurons (Figures 4A and 4B). Antidromicity was determined by collision tests and by response to two electrical pulses at high frequency (Figures 4A and 4B). In vivo recordings of amBNST neurons projecting to the VTA demonstrated that $HFS_{vSUB}$ promoted a potentiation of the excitatory responses evoked by vSUB electrical stimulation (Rmag = 185.61% ± 21.28% of the baseline; mean average 20–40 min after $HFS_{vSUB}$; n = 6; $p < 0.01$; Figures 4C and 4D). In addition, this potentiation was dependent on activation of NMDA subtype of glutamate receptors (90.94% ± 25.12% of the baseline; n = 4; N.S.; Figures 4C and 4D). To test whether NMDA receptor activity in the amBNST in response to $HFS_{vSUB}$ gates the hyperactivity of the tonic firing parameters of the VTA dopamine neurons, we infused the NMDA antagonist AP5 into the amBNST prior to inducing $HFS_{vSUB}$ and recorded the activity of VTA dopamine neurons (Figure 5) at two time points, i.e., 1 and 5 days after the infusion. Infusion of the NMDA antagonist AP5 into the amBNST prior to inducing $HFS_{vSUB}$ prevented the hyperactivity of the VTA dopamine neurons 1 and 5 days later (Figure 5). One day after AP5/$HFS_{vSUB}$ treatment, the bursting rate and the percentage of spikes in burst decreased in the $HFS_{vSUB}$ group compared to the SHAM group (bursting activity: SHAM group: 0.28 ± 0.04 Hz; 1D-$HFS_{vSUB}$: 0.17 ± 0.03 Hz; $p < 0.01$; spike in burst: SHAM group: 22.68 ± 3.06 Hz; 1D $HFS_{vSUB}$: 15 ± 2.12 Hz; $p < 0.05$; Figures 5B–5E). Five days after AP5/$HFS_{vSUB}$ treatment, the firing rate was decreased in the $HFS_{vSUB}$ group compared to the SHAM group (firing rate: SHAM group: 4.56 ± 0.38 Hz; 5D-$HFS_{vSUB}$: 3.03 ± 0.39 Hz; $p < 0.01$; Figures 5F–5I). Altogether, these results strongly suggest that the in vivo NMDA-based LTP in $amBNST_{\rightarrow VTA}$ neurons induced by $HFS_{vSUB}$ gates in vivo the hyperactivity of VTA dopamine neurons.

## vSUB Stimulation Potentiates the Behavioral Effect of Cocaine Administration

Cocaine exerts its locomotor effect through an increased dopaminergic transmission in the Nac (Robinson and Berridge, 2001). It acts as a dopamine re-uptake inhibitor, but the enhanced release of dopamine by cocaine requires spiking activity of VTA dopamine neurons (Nomikos et al., 1990). In agreement with a role of VTA dopamine neuron tonic activity in the physiological and behavioral effect of cocaine (Sombers et al., 2009), we tested the impact of $HFS_{vSUB}$ on locomotor response to cocaine challenge. Electrophysiological findings suggested that $HFS_{vSUB}$-induced NMDA-dependent LTP in the amBNST is permissive for the potentiation of tonic activity of VTA dopamine neurons. If true, then $HFS_{vSUB}$ should modulate behavioral response to cocaine in an amBNST-dependent manner. The NMDA antagonist AP5 (or vehicle, aCSF) was infused into the amBNST and locomotor activity in response to a sub-threshold dose of cocaine (7.5 mg/kg, i.p.), i.e., a dose that does not elicit increased locomotion per se, was measured 5 days after $HFS_{vSUB}$ or SHAM (Figure 6A). We performed histological controls to ensure that we exclusively targeted the amBNST with the AP5 injection and not the neighboring Nac (Figure 6B). A global ANOVA with repeated measures indicated differences in ambulatory activity between all groups (HFS × AP5 × coc interaction; $F_{(1, 20)} = 6.25$; $p < 0.02$). There was no difference in the locomotor activity in response to saline whatever the stimulation protocol and the BNST treatment (HSF × AP5 interaction; $F_{(1, 20)} = 0.23$; $p > 0.05$; SHAM 33.5 ± 2.9 crossings, $HFS_{vSUB}$ 31.5 ± 3.6 crossings; SHAM AP5 21.7 ± 4.1 crossings, $HFS_{vSUB}$ AP5 16.2 ± 3.9 crossings; Figure 6C). As expected, the challenge with a sub-threshold dose of cocaine did not significantly

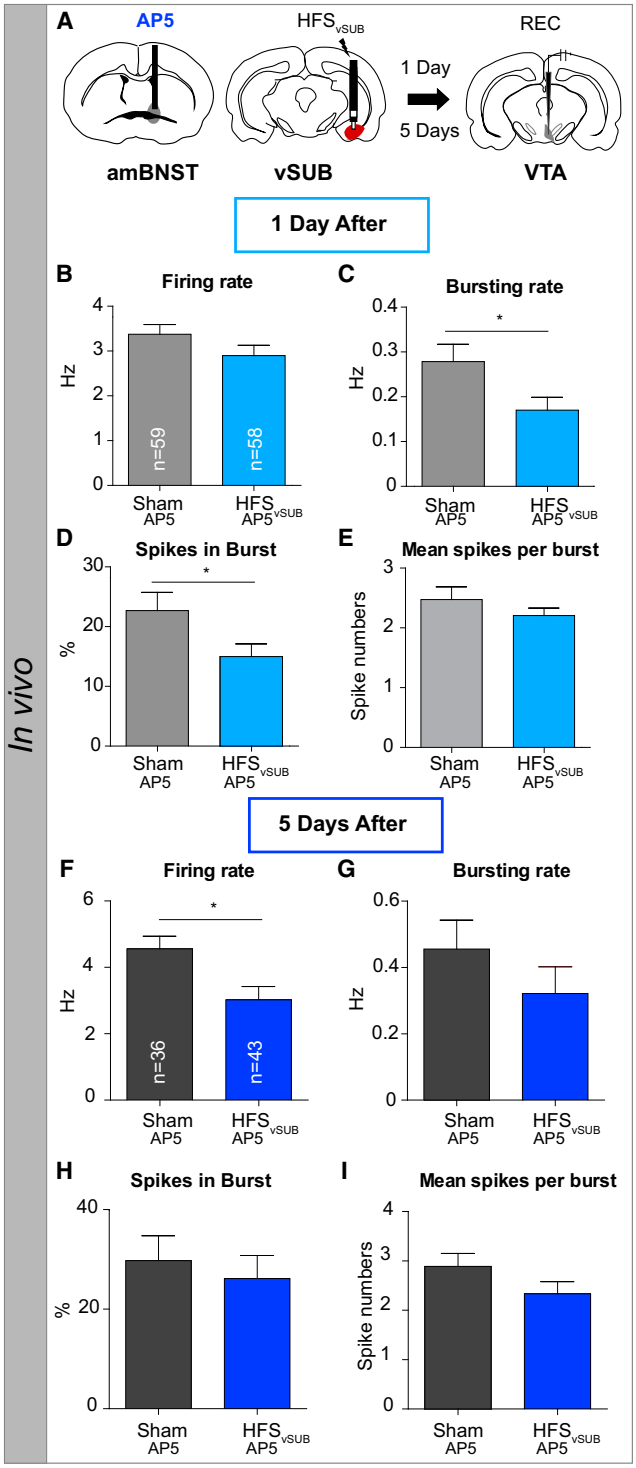



**Figure 5. LTP in amBNST Neurons Is Permissive for VTA Dopamine Potentiation**

(A) Experimental protocol.

(B–E) Graphs illustrating the effect of AP5 injection into the amBNST followed by $HFS_{vSUB}$ (blue, $HFS_{vSUB}$) or without stimulation (gray, SHAM $_{AP5}$) on VTA dopamine neuron tonic firing activity (B) and bursting parameters (C–E), 1 day after.

(F–I) Graphs illustrating the effect of AP5 injection into the amBNST followed by $HFS_{vSUB}$ (dark blue, $HFS_{vSUB}$) or without stimulation (dark gray, SHAM $_{AP5}$) on VTA dopamine neuron tonic firing activity (F) and bursting parameters (G–I), 5 days after.

change locomotor activity in the $SHAM_{vSUB}$ group (SHAM saline versus SHAM cocaine: 54.5 ± 18.4 crossings; LSD Fisher $p > 0.05$). In SHAM AP5-treated animals, the same cocaine challenge increased locomotor activity (SHAM AP5 saline versus SHAM AP5 cocaine: 80.7 ± 13.1 crossings; $p < 0.002$). However, $HFS_{vSUB}$ potentiated the locomotor response to cocaine ($HFS_{vSUB}$ saline versus $HFS_{vSUB}$ cocaine: 124.2.0 ± 21.5 crossings; LSD Fisher $p < 0.00002$). Importantly, this effect was blocked if rats were previously injected with the NMDA antagonist AP5 in the amBNST ($HFS_{vSUB}$ cocaine versus $HFS_{vSUB}$ cocaine AP5: 61.2 ± 10.7 crossings; LSD Fisher $p < 0.0007$). These data show that $HFS_{vSUB}$-driven amBNST-dependent hyperactivity of dopamine neuron is permissive for the expression of cocaine-induced locomotion.

## DISCUSSION

Whereas a role for the vSUB in controlling the VTA dopamine neurons has been clearly established (Floresco et al., 2001b; Legault et al., 2000; Legault and Wise, 1999; Todd and Grace, 1999), the circuit-based mechanism by which glutamate activates VTA dopamine neurons after stimulation of the vSUB was still unknown. Here, we characterized in vivo the neural network underlying the persistent hyperactivity of dopamine neurons in response to $HFS_{vSUB}$. First, we show that vSUB stimulation induces phasic glutamatergic excitatory responses in VTA dopamine neurons (Figure 1). These data are in accordance with a pioneering microdialysis study using infusion of glutamatergic antagonists in the VTA to demonstrate the involvement of VTA glutamatergic transmission in the vSUB-evoked increase of dopamine release in the Nac (Legault et al., 2000). We also show that the amBNST relays the glutamatergic drive between the vSUB and dopamine neurons of the VTA. Our results demonstrate the existence of a pathway vSUB-VTA parallel to the one previously described (Floresco et al., 2001a; Todd and Grace, 1999). Indeed, it has been previously reported that the vSUB controls the proportion of spontaneously active DA neurons without affecting their firing or bursting activity through a disinhibitory mechanism involving the Nac and the ventral pallidum (Floresco and Phillips, 2001). Here, we propose an alternative polysynaptic pathway where the amBNST relays information between the vSUB and the VTA. Moreover, we observed that the number of cells per track, which is thought to reflect the level of spontaneous activity, was increased 1 day after the $HFS_{vSUB}$ but was unaffected by the infusion of glutamatergic antagonists in the amBNST (data not shown). This observation suggests that the vSUB-Nac pathway controls the proportion of spontaneously active VTA dopamine neurons (Floresco et al., 2001a), whereas the firing and bursting activities are regulated by the vSUB-amBNST pathway. In addition to these excitatory responses, our data demonstrate that electrical stimulation of the vSUB produces inhibitory responses in VTA dopamine

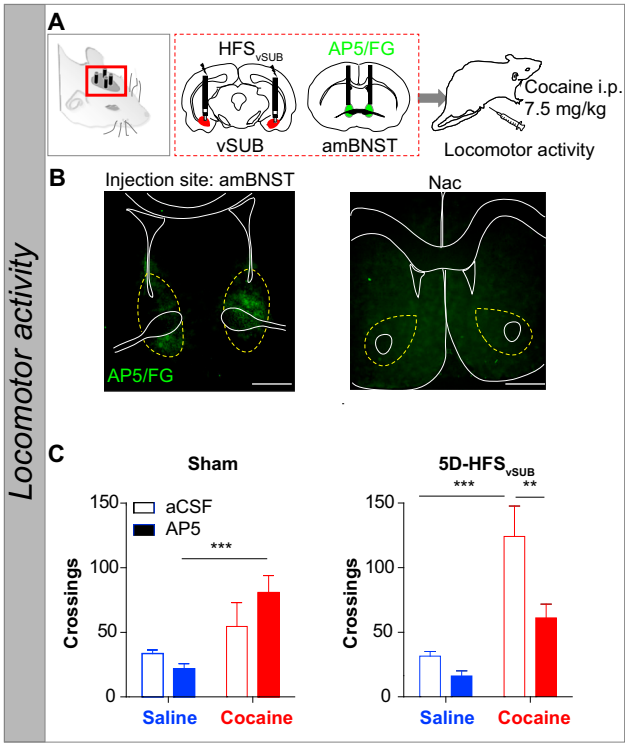



**Figure 6. $HFS_{vSUB}$ Potentiates Behavioral Effect of Cocaine in an amBNST-Dependent Manner**

(A) Experimental protocol.

(B) Histological control of the injection site of AP5/FG: in the amBNST. All the solutions infused into the amBNST were diluted in 0.2% Fluorogold (FG) in order to monitor the diffusion of the injection. The absence of fluorescence in the nucleus accumbens (Nac) showed that the infused solutions in the amBNST were not diffusing in the Nac. The scale bars represent 0.5 mm.

(C) Graphs showing locomotor activity in response to a low dose of cocaine (7.5 mg/kg, i.p., or saline) measured 5 days after $HFS_{vSUB}$ or SHAM associated to AP5 (or vehicle, aCSF) infusion into the amBNST.

neuron insensitive to the blockade of VTA glutamatergic transmission (Figures 1F, 1I, and 1J). This observation suggests that VTA feedforward inhibition is not involved in the inhibitory response. Further investigation is needed to clarify the distinct sub-circuit supporting inhibitory responses, but because both GABAergic and glutamatergic projections from the BNST to the VTA have been identified (Georges and Aston-Jones, 2001; Jennings et al., 2013; Kudo et al., 2012), the BNST-VTA pathway could be a potential candidate. Additional pathways supporting the inhibitory responses evoked by the stimulation of the vSUB could involve a relay into the Nac (Floresco et al., 2001a) or a long-range inhibitory projection from the hippocampus (Jinno et al., 2007).

Additionally, $HFS_{vSUB}$ induced an in vivo hyperactivity of the tonic firing parameters of VTA dopamine neurons 1 day later. This late potentiation was maintained for 5 days but was absent in the first hour following the $HFS_{vSUB}$ (Figure S3). Previous studies have reported that an in vivo single exposure to cocaine results in an increase in the AMPA- to NMDA-receptor-mediated EPSCs ratio in VTA dopamine neurons (Saal et al., 2003; Ungless et al., 2001). The AMPA receptor redistribution in dopamine neurons is driven by the concomitant increase in glutamatergic transmission and activation of dopamine receptors within the VTA (Schilström et al., 2006; Ungless et al., 2001). Presumably, the enhancement of glutamatergic transmission and release of dopamine in the VTA after vSUB stimulation (Legault et al., 2000; Legault and Wise, 1999, 2001; Taepavarapruk et al., 2008) trigger both the increase in AMPA/NMDA ratio (Figure S2) and the hyperactivity of VTA dopamine neurons. However, we cannot rule out the possibility that these synaptic and spiking adaptive changes in VTA dopamine neurons develop in parallel and are not causally connected.

Here, we demonstrate that a single cocaine challenge with a sub-threshold dose affected locomotor activity differently depending on whether animals had received an $HFS_{vSUB}$ or sham stimulation. This facilitation of cocaine-evoked locomotor activity depends on NMDA receptor activation within the amBNST. Motivation and reward-related behaviors are largely mediated by the amBNST (Jennings et al., 2013; Krawczyk et al., 2013; Reisiger et al., 2014). A current hypothesis is that brain circuits normally used in motivational behavior and/or in learning and memory processes are remodeled following drug consumption. Our results show that a physiological synaptic remodeling of the vSUB-amBNST-VTA circuit gates the in vivo hyperactivity of VTA dopamine neurons in a time-dependent manner and facilitates locomotor activity induced by a sub-threshold dose of cocaine. Thus, these findings confirm the substantial role of the vSUB in drug sensitization (Goto and Grace, 2005; Lodge and Grace, 2008) and suggest that a unique $HFS_{vSUB}$, inducing persistent NMDA-dependent LTP in $amBNST_{\rightarrow VTA}$ neurons, could mimic the synaptic changes developing over time during repeated cocaine exposure and leading to locomotor sensitization (Robinson and Berridge, 1993). Together, these findings provide further insights into the known circuitry regulating VTA dopamine neurons and highlight important future therapeutic directions where manipulation of the vSUB-amBNST-VTA pathway could play a role in erasing drug-associated memory.

## EXPERIMENTAL PROCEDURES

### Animals

Sprague Dawley rats (275–300 g; Janvier Lab) were used. Rats were housed three or four per cage under controlled conditions (22°C–23°C; 40% relative humidity; 12 hr light/dark illumination cycle; lights on from 7:00 a.m. to 7:00 p.m.) and were acclimatized to laboratory conditions 1 week before the experiment with food/water ad libitum. All procedures were conducted in accordance with the European directive 2010-63-EU and with approval from Bordeaux University Animal Care and Use Committee (no. 50120205-A).

### Surgery

Stereotaxic surgeries for electrophysiology were performed under isoflurane anesthesia as previously described (Georges and Aston-Jones, 2002). Stimulation electrodes, recording pipettes, or ejecting pipettes were inserted into the vSUB (−6.0 mm/bregma; 5.1 mm/midline; 7.1 mm/brain surface), the amBNST (0.0 mm/bregma; 1.3 mm/midline; 6–7.5 mm/brain surface), or the VTA (−5.3 mm/bregma; 0.7 mm/midline; 7.8–8.5 mm/brain surface), respectively.

### Electrical Stimulation of the vSUB

Bipolar electrical stimulation of the vSUB was performed with a concentric electrode (Phymep) and a stimulus isolator (500 μs duration; 0.2–1 mA;

Digitimer). Baseline was recorded for 10 min (2 × 100 pulses; 0.5 Hz). High-frequency stimulation was performed in the vSUB ($HFS_{vSUB}$) (0.1–1 mA; same intensity use as that used for the baseline). $HFS_{vSUB}$ consisted in 50 trains (500 pulses at 400 Hz; 250 μs duration pulse) presented as bursts of five trains. The frequency of the five trains was 1 Hz. Each burst of five trains was presented five times at 1 min interval. Only one cell per rat was recorded for neuroplastic responses.

### Pharmacological Treatment

Double-barrel pipettes (Georges and Aston-Jones, 2002) were used to record VTA dopamine or amBNST spike activities while simultaneously microinjecting one of the following: a mixture of 100 μM amino-5-phosphonopentanoic acid (AP5) and 50 μM 6-cyano-7nitroquinoxaline-2,3-dione (CNQX) or artificial cerebrospinal fluid (aCSF). All drugs were dissolved in aCSF. For the behavioral part, we injected locally into the amBNST (bilateral injections) 180 nl of a mixture of AP5 (100 μM) and 0.2% Fluorogold (in order to monitor the diffusion of the injection).

### Tract-Tracing Method

Tracer injections were performed as described previously (Jalabert et al., 2009) with the following modifications. For retrograde tracing, 30 nl of 0.5% CTb (Sigma Aldrich) were infused by pressure, in the VTA (n = 5). Animals received a single iontophoretic injection of a 2.5% solution of an anterograde tracer PHA-L (Vector Laboratories) in the vSUB (n = 3). Animals were allowed to survive 7–14 days.

### In Vivo Single-Unit Neuron Recordings

A glass micropipette (tip diameter: 2–3 μm; 4–6 MΩ for VTA dopamine neurons; 1–2 μm; 10–15 MΩ for amBNST neurons) filled with 2% pontamine sky blue solution in 0.5 M sodium acetate was lowered into the VTA or amBNST. Glass electrodes were lowered in the anterior and lateral part of the VTA according to stereotaxic coordinates derived from rat brain atlas and corrected empirically (antero-posterior: −5.0–6.0 mm from bregma; mediolateral: 0.7–1.0 mm from midline; dorso-ventral: −7.8–8.4 mm from bregma). To distinguish dopamine from non-dopamine neurons, the following parameters were used: (1) an action potential width ≥1.1 ms (measured from the start of action potential to the negative trough); (2) slow spontaneous firing rate (<10 Hz); (3) single and burst spontaneous firing patterns (characterized by spike-amplitude decrement); (4) inhibition of spontaneous activity by DA receptor agonists and subsequent reversal by DA receptor antagonists (data not shown). Neurochemical identification through single-cell labeling and immunohistochemistry (Jalabert et al., 2011) confirmed that the use of the electrophysiological criteria was extremely reliable for the online identification of dopamine neurons. The extracellular potential was recorded with an Axoclamp-2B amplifier and filter (300 Hz/0.5 Hz; Georges and Aston-Jones, 2002). Single-neuron spikes were collected online (CED 1401; SPIKE 2; Cambridge Electronic Design). During electrical stimulation of the vSUB, cumulative peristimulus time histograms (PSTHs) (5-ms bin width) of VTA or amBNST activity were generated for each neuron recorded. Electrical stimulation of the VTA was used to test for antidromic activation of amBNST neurons ($amBNST_{\rightarrow VTA}$ neurons) using high-frequency following and collision methods, as previously described (Georges and Aston-Jones, 2002). A bipolar concentric stimulation electrode was inserted into the VTA, and stimulation of the VTA and recordings in the amBNST using methods were performed as described above. Driven impulses were considered antidromic if they met the following criteria: (1) constant latency of spike response, (2) driven by each of the paired stimulus pulses at frequencies of 100 Hz or greater, and (3) collision of driven spikes by spontaneous impulses occurring within a critical interval approximately equal to the sum of the refractory period plus the driving latency. A measure of the axonal refractory period was obtained by determining the minimum interval between paired stimuli producing two action potentials 100% of the time. The delay at which the second response disappeared corresponded to the absolute refractory period of that particular unit. Absolute refractory period measurement and collision of driven spikes with spontaneous impulses were conducted with stimulation amplitudes of 1.5- to 2.0-fold the threshold for driving.

### In Vitro Patch-Clamp Recordings

Rats from the SHAM group or the $HFS_{vSUB}$ group were anesthetized (ketamine/xylazine) 5 days after $HFS_{vSUB}$ for preparation of VTA-containing brain slices. Slicing was performed in bubbled ice-cold 95% O2/5% CO2-equilibrated solution containing the following (in mm): choline Cl 110; glucose 25; NaHCO3 25; MgCl2 7; ascorbic acid 11.6; $Na^+$-pyruvate 3.1; KCl 2.5; NaH2PO4 1.25; and CaCl2 0.5 and recovered for 10 min in warm artificial CSF containing the following (in mm): NaCl 124; NaHCO3 26.2; glucose 11; KCl 2.5; CaCl2 2.5; MgCl2 1.3; and NaH2PO4 1.A. Horizontal slices (250 μm) were stored at room temperature in 95% O2/5% CO2-equilibrated artificial CSF. Recordings (flow rate of 2.5 ml/min) were made under an Olympus BX51 microscope at 32°C. Currents were amplified, filtered at 5 kHz, and digitized at 20 kHz. Access resistance was monitored by a −10-mV step (0.1 Hz). Experiments were discarded if the access resistance increased >20%. The internal solution contained the following (in mm): CsCl 130; NaCl 4; MgCl2 2; EGTA 1.1; HEPES 5; Na2ATP 2; $Na^+$-creatine-phosphate 5; and Na3GTP 0.6, and spermine 0.1 was added when indicated. Dopamine neurons located in the VTA identified as the region medial to the medial terminal nucleus of the accessory optic tract were identified by their large capacitance (>40 pF) and by the presence of Ih. The liquid junction potential was −3 mV. EPSCs were pharmacologically isolated in the presence of picrotoxin (100 μM) and evoked through glass electrodes placed rostral to the patched cell. AMPA/NMDA ratios of evoked EPSC were obtained by AMPA-EPSC +40 mV/NMDA-EPSCs at +40 mV. The NMDAR component was calculated as the difference between the EPSCs measured in the absence and presence of D,L-AP5.

### Locomotor Activity Recording

Locomotor ambulatory activity was measured in activity cages (35 × 25 × 25 cm) with wire-mesh floors and 10-mm Plexiglas sidewalls (Imetronic) and expressed in crossings. Two infrared photoelectric cells were placed 14 cm apart and 3 cm above the floor. The activity cages were kept in a dimly lit room with a continuous white noise. Each experiment started with a 30-min habituation period, in which rats were placed in the activity chamber without any injection.

### Locomotor Response to Cocaine Challenge

Animals were assigned to four groups according to the vSUB stimulation protocol and the intra-amBNST pharmacological pretreatment ($HFS_{vSUB}$ + aCSF, $HFS_{vSUB}$ + AP5, SHAM + aCSF, and SHAM + AP5). Intra-amBNST aCSF (120 nl) or AP5 (120 nl; 100 μM) were infused 2 or 3 min prior to inducing $HFS_{vSUB}$ or sham manipulation (SHAM). All the solutions infused into the amBNST were diluted in 0.2% Fluorogold (in order to monitor the diffusion of the injection). Rats were tested for the locomotor response to cocaine (7.5 mg/kg/ml, i.p.) as follows: first, rats were injected with saline (1 ml/kg, i.p.) and then monitored for 30 min in the activity cages. Immediately after, they were injected with cocaine and monitored for 30 min.

### Histology

At the end of each recording experiment, the recording pipette placement was marked with an iontophoretic deposit of pontamine sky blue dye (−20 μA; 30 min). To mark the electrical stimulation sites, +50 μA was passed through the stimulation electrode for 90 s. Then brains were removed, snap frozen in a solution of isopentane, and stored at −80°C.

### Immunohistochemical Methods

Immunocytochemical detection was performed by standard light, fluorescent microscopy as previously described (Georges and Aston Jones 2002). Rats were perfused transcardially (4% paraformaldehyde solution). Sections were incubated (overnight/4°C) with rabbit anti PHAL primary antibody (1/2,000; Clinisciences), goat anti CTb primary antibody (1/10,000; List Biological Laboratories). Following washes, in one set of experiment, sections were incubated overnight at 4°C with biotinylated donkey antirabbit secondary antibody (1/200; Millipore) and biotinylated donkey antigoat secondary antibody (1/200; Millipore), followed by an avidin/biotin complex (kit ABC; Vector Laboratories). Staining was revealed after an incubation in diaminobenzidine/nickel solution (Vector Laboratories). For fluorescence, sections after washes were incubated overnight at 4°C with donkey antirabbit secondary antibody (labeling of PHAL;

1/1,000; Invitrogen; Alexa Fluor 568), donkey antigoat secondary antibody (labeling of CTb; 1/1,000; Jackson Immunoresearch; Alexa Fluor 647), or donkey antigoat secondary antibody (labeling of PHAL; 1/1,000; Invitrogen; Alexa Fluor 568). Tissue sections were washed and then mounted in Vectashield medium (Vector Laboratories), coverslipped and imaged on a fluorescent microscope. Photomicrographs were taken and displayed using Image J.

### Data Analysis

For in vivo electrophysiological experiments, four parameters for VTA dopamine neurons firing and bursting activity were analyzed: firing rate; bursting rate; the proportion of spikes in the burst; and the mean number of spikes per burst. Cumulative PSTHs of VTA or amBNST activity were generated during stimulation of vSUB or VTA. Excitatory magnitudes ($R_{mag}$ values) were normalized for different levels of baseline impulse activity. $R_{mag}$ values for excitation and inhibition were calculated according to $R_{mag}$ = (counts in excitatory epoch) − (mean counts per baseline bin × number of bins in excitatory epoch). The hippocampal excitation strength on amBNST neurons was determined as the amount of current needed to obtain a 50% spike probability of vSUB-evoked responses ($R_{mag}$ ranging from 30 to 60). Results are expressed as mean ± SEM.

For statistic, two group comparisons were performed with Student's t tests. For multiple comparisons, values were subjected to a one-way ANOVA followed by Newman Keuls post hoc test or to ANOVA with repeated measures followed by LSD Fisher post hoc test for the behavioral part.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.076.

## AUTHOR CONTRIBUTIONS

F.G., S.C., M.M., P.F., and M.D. designed the project. C.G., G.R.F., M.J., and F.G. designed, performed, and analyzed the in vivo electrophysiological data. S.L., K.V., F.J.M., and M.M. designed, performed, and analyzed the electrophysiology in acute slices. S.C. designed, performed, and analyzed the behavioral data. F.G. and C.G. wrote the manuscript. All authors discussed the results and commented the manuscript.

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