

# The amygdala-ventral pallidum pathway contributes to a hypodopaminergic state in the ventral tegmental area during protracted abstinence from chronic cocaine

Adelie Salin<sup>1</sup>, Emilie Dugast<sup>1</sup>, Virignie Lardeux<sup>1</sup>, Marcello Solinas<sup>1</sup>, and pauline belujon<sup>1</sup>

<sup>1</sup>University of Poitiers

September 8, 2022

## Abstract

**Background and Purpose** Incubation of craving is associated with temporal changes in the activity of several structures involved in drug-seeking behavior. Hypodopaminergic activity, responsible for negative emotional states, has been reported in the ventral tegmental area (VTA) during cocaine abstinence. The neuroadaptations underlying the VTA hyperdopaminergic state after chronic cocaine is not well understood. In this work, we investigated the potential involvement of a VTA inhibiting circuit (amygdala-ventral pallidum (VP) pathway) in the hypodopaminergic state during abstinence from chronic cocaine. **Experimental Approach** In a model of cocaine self-administration, we performed in vivo electrophysiological recordings of DA VTA neurons and basolateral amygdala (BLA) neurons from anesthetized rats during early and protracted abstinence and evaluated the involvement of the BLA-VP pathway using a pharmacological approach. **Key Results** We found a significant decrease of VTA DA population activity and a significant increase of BLA activity after 30 days of abstinence from chronic cocaine but not one day. The decrease in VTA DA activity was restored by pharmacological inhibition of the activity of either the BLA or the VP. **Conclusion and Implications** Our study sheds new lights on neuroadaptations occurring during incubation of craving leading to relapse. In particular, we described the involvement of the BLA-VP pathway in cocaine-induced decreases of DA activity in the VTA. This study adds an important building block to the characterization of specific brain network dysfunctions underlying hypodopaminergic activity during abstinence.

## Title

The amygdala-ventral pallidum pathway contributes to a hypodopaminergic state in the ventral tegmental area during protracted abstinence from chronic cocaine

## Running title

A circuit regulating cocaine-induced hypodopaminergic state

## Authors

Salin Adélie<sup>1,3</sup>, Dugast Emilie<sup>1,2</sup>, Lardeux Virginie<sup>1</sup>, Solinas Marcello<sup>1</sup> and Belujon Pauline<sup>1</sup>

## Affiliation

<sup>1</sup> Université de Poitiers, INSERM, U-1084, Laboratoire des Neurosciences Expérimentales et Cliniques, Poitiers, France

<sup>2</sup> CHU de Poitiers, Poitiers, France

<sup>3</sup>new address: Université de Rennes, Institut Numecan INRAE, INSERM, Rennes, France

## Word count

Abstract: 245 words

Main text: 2571 words

References: 49

## Acknowledgements

This work has benefited from the facilities and expertise of PREBIOS platform (University of Poitiers). This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National pour la Recherche Scientifique, the University of Poitiers, CHU of Poitiers, SFR FED 4226 and the Agence Nationale de la Recherche (ANR JC, ANR-15-CE37-0010 to P.B.).

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

## Conflict of Interest statement

The authors declare no potential conflict of interest

## Abstract

### Background and Purpose

Incubation of craving is associated with temporal changes in the activity of several structures involved in drug-seeking behavior. Hypodopaminergic activity, responsible for negative emotional states, has been reported in the ventral tegmental area (VTA) during cocaine abstinence. The neuroadaptations underlying the VTA hyperdopaminergic state after chronic cocaine are not well understood. In this work, we investigated the potential involvement of a VTA inhibiting circuit (amygdala-ventral pallidum (VP) pathway) in the hypodopaminergic state associated with abstinence from chronic cocaine.

### Experimental Approach

In a model of cocaine self-administration, we performed *in vivo* electrophysiological recordings of DA VTA neurons and basolateral amygdala (BLA) neurons from anesthetized rats during early and protracted abstinence and evaluated the involvement of the BLA-VP pathway using a pharmacological approach.

### Key Results

We found a significant decrease of VTA DA population activity and a significant increase of BLA activity after 30 days of abstinence from chronic cocaine but not after one day. The decrease in VTA DA activity was restored by pharmacological inhibition of the activity of either the BLA or the VP, suggesting that these regions exert a negative influence on DA activity.

### Conclusion and Implications

Our study sheds new lights on neuroadaptations occurring during incubation of craving leading to relapse. In particular, we described the involvement of the BLA-VP pathway in cocaine-induced decreases of DA activity in the VTA. This study adds an important building block to the characterization of specific brain network dysfunctions underlying hypodopaminergic activity during abstinence.

## Highlights

### What is already known

Protracted abstinence from cocaine self-administration produces hyperactivity of the BLA and hypoactivity of VTA DA neurons

The BLA-ventral pallidum pathway is a potent inhibitory circuit of VTA DA activity.

## What the study adds

Hyperactivity of the BLA and hypoactivity of VTA DA neurons is not present during early abstinence but requires several weeks to develop.

Increased activity of the BLA-VP pathway inhibits DA activity after long-term abstinence

## What is the clinical significance

Hypodopaminergic activity after prolonged abstinence might underlie dysphoria leading to relapse

Imaging studies of the circuit regulating DA activity might give critical information on emotional symptoms leading to relapse.

## Key words

Dopamine, cocaine, amygdala, incubation of craving, ventral pallidum, relapse, dysphoria

## Main text

### Introduction

Chronic cocaine produces persistent neuroadaptations in brain networks that underlie long-term risks of relapse and incubation of drug craving (van Huijstee and Mansvelder, 2015). Rewarding properties of psychostimulants such as cocaine are associated with hyperdopaminergic state (Wise and Hoffman, 1992) whereas acute withdrawal from acute and chronic psychostimulants leads to a hypodopaminergic state, specifically in the mesolimbic system (Shen et al., 2007; Belujon et al., 2016; Salin et al., 2021), leading to hedonic deficits (Wise, 2008; Belujon and Grace, 2017). Reduction of DA activity is also observed after long abstinence (Diana et al., 1996; Salin et al., 2021); this reduction could be involved in dysphoria described after protracted abstinence (Haake et al., 2019).

DA activity is regulated by different afferent circuits. In particular, the basolateral nucleus of the amygdala (BLA)-ventral pallidum (VP) pathway has been shown to inhibit VTA DA activity (Chang and Grace, 2014). The BLA is a crucial brain structure involved in emotional processes (LeDoux, 2000; Adhikari et al., 2015; Janak and Tye, 2015) and in reward-associated learning and memory (Murray, 2007; Wassum and Izquierdo, 2015). In drug addiction, the BLA is a major brain substrate for drug-associated cue memory and imaging studies in humans report increased activity in the amygdala in cocaine craving addicts exposed to drug-cues (Maas et al., 1998; Childress et al., 1999; Garavan et al., 2000). In rodents, increased activity of BLA neurons have been described during abstinence from chronic cocaine (Munshi et al., 2019) and exposure to drug-related environmental cues triggers drug-seeking behavior which is alleviated by lesion of the BLA (Meil and See, 1997). The VP is a central site for limbic reward signals (Smith et al., 2009) and is involved in drug-seeking behavior after long-term abstinence (Farrell et al., 2019), in particular its projections to the VTA (Mahler et al., 2014). Importantly, the BLA-dependent decrease in VTA activity found in psychiatric disorders such as major depressive disorders appears to be regulated by the VP (Chang and Grace, 2014). However, it is not known whether the same circuit plays a role in DA hypoactivity that characterize prolonged abstinence.

In this work, we investigated the potential involvement of the BLA and the VP in VTA hypodopaminergic activity during withdrawal from chronic cocaine, using a model of cocaine self-administration, *in vivo* electrophysiological recordings of DA VTA neurons and BLA putative projections neurons from anesthetized rats during early and protracted abstinence.

## Methods

### Animals

Male Sprague Dawley rats (Charles River Laboratories, France) weighing 325-350g upon arrival, were housed in a temperature and humidity-controlled environment and maintained on a 12-h light/12-h dark cycle (on at 07:00 am). Animals were housed two per cage from arrival to surgery and one per cage after surgery.

Experiments were performed during the light phase. Animals had *ad libitum* access to food and water in the homecage. All experiments were conducted in accordance with the European Union directives (2010/63/EU) for the care of laboratory animals and all experimental protocols were approved by the local ethics committee (COMETHEA, n°7147).

## Cocaine Self-Administration and Seeking

*Catheter implantation.* Rats were prepared for cocaine self-administration by surgical catheterization of the right jugular vein. Briefly, rats were anesthetized with isoflurane (5% induction, 2.5% maintenance) in O<sub>2</sub> and administered with the nonsteroidal anti-inflammatory ketoprofen (2.5mg/kg, *s.c.*). Animals were placed on a heating pad from the induction of the anesthesia to the end of the surgery. A handmade silastic catheter was inserted into the jugular vein and the distal end was led to the back between the scapulae. Rats were allowed to recover for 7 days and flushed daily with 0.1ml sterile saline (0.9%), gentamicin (20mg/ml) and heparin (100 UI/mL) in sterile saline to help protect against infection and catheter occlusion.

*Apparatus.* Experiments were conducted in operant-conditioning chambers, equipped with nose-pokes as *operanda*, a cue-light above the active nose-poke, a house light and controlled by Imetronic interfaces and software (Imetronic, Pessac, France).

*Cocaine self-administration procedure.* Rats were allowed to self-administer cocaine (Cooper, France; 6g/L in saline solution 0.9%; 0.75mg/kg/infusion). Control rats received saline infusions according to a yoked procedure, which consists of the delivery of an injection of saline each time the paired « master » rat self-administered an injection of cocaine. Rats were placed in the self-administration chamber for 6h/day for ten sessions, using a Fixed Ratio 1 (FR1) schedule of reinforcement. Response in the active nose poke resulted in one intravenous (*i.v.*) cocaine infusion with the concomitant activation of the light that remained on for 5s and then pulsed for 5s, followed by a 5s time-out. Inactive nose-pokes were recorded but did not produce any consequences. After the last self-administration session, rats remained in their homecage for up to a month of forced abstinence. Animals were then either used for electrophysiological recordings or tested for cocaine-seeking behavior.

*Cocaine-seeking behavior.* Abstinent cocaine rats were re-exposed to the operant chambers after 1 and 30 days of abstinence as previously done (Chauvet et al., 2012). Active nose-pokes triggered cue-light and syringe holder activation (similarly to self-administration sessions), but rats were not connected to tubing and did not receive any cocaine infusions. Cocaine-seeking behavior was measured for 1 h where active and inactive responses were recorded. The same groups of rats were used for each time point.

## In Vivo Single-Unit Extracellular Recordings

*Local drug administration.* A cannula was lowered into targeted regions at the following coordinates on the right hemisphere (AP relative to bregma, ML relative to midline, DV relative to brain surface): BLA; AP -2.8 mm, ML +4.8 mm, DV -6.8 mm / VP; AP -0.3 mm, ML +2.0 mm, DV -6.8 mm. Drug solution were prepared with Dulbecco's PBS Buffer: TTX, 25 nmol/0.5  $\mu$ L/infusion (Tocris, CAS 4368-28-9), kynurenic acid (5  $\mu$ g in .5  $\mu$ L) (Sigma, CAS 492-27-3). Kynurenic acid, a glutamate receptor antagonist, is known to block glutamatergic inputs onto VP (Chang and Grace, 2014). Drugs or vehicle were infused 1 min after cannula lowering by slow manual infusion. Cannula was left in place for an additional 1 minute and then removed before VTA DA neurons recordings.

For acute withdrawal, extracellular recordings were performed 18h up to 72h after the last cocaine self-administration session. For protracted withdrawal, recordings were performed from 26 days to 45 days after the last session. Naïve rats have also been used for recording of DA activity. Since there was no difference in DA activity between naïve rats and saline WD1-3 and saline WD26-45, data have been pooled.

*Single-unit extracellular recordings.* Rats were anesthetized with isoflurane (5 % induction, 2.5 % maintenance) and placed in a stereotaxic frame. Body temperature was maintained at 37 °C with a temperature-controlled heating pad. The scalp was exposed and burr holes were drilled in the skull overlying the VTA, the BLA or the VP. Extracellular recording electrodes were pulled from glass micropipettes (WPI; impedance



10-20 M $\Omega$  for BLA recordings, 6-8 M $\Omega$  for VTA recording). The tip of the glass microelectrode was broken to a diameter of 2  $\mu$ m, and filled with a 2 % Chicago Sky Blue dye (Sigma, CAS 2610-05-01) solution in 2 M NaCl.

For DA neurons, recording electrode was lowered using a microdrive through the right VTA in 9 sequential vertical tracks separated by 0.2mm (Supplementary figure 1). Spontaneously active cells encountered identified as DA neurons (online analysis) were recorded to determine population activity as previously described (Salin et al., 2021). Single-unit activity recorded from the VTA was amplified 10 times, filtered (low pass: 30 Hz; high pass: 16 kHz), and further amplified 50 times (Multiclamp 700b, Axon Instruments, Union City, CA). The signal was digitized at 16 kHz (CED 1401) and acquired on a computer using Spike 2 7.0 software (Cambridge Electronics Design, Cambridge, UK). Three parameters were analyzed: the number of neurons/electrode track, firing rate and the percentage of spikes in bursts.

For BLA recordings, electrode was lowered through the right BLA at the following coordinates: AP -2.8mm to -3.2mm (from bregma), ML +4.6mm to 5.2mm (from midline), DV -7.0mm to -9.5 (from brain surface). Encountered cells in the targeted area meeting BLA putative pyramidal neuron's criteria (action potential from peak to trough [?] 0.5ms (Bienvenu et al., 2012) and with a signal-to-noise ratio of 3:1 (or greater) were recorded for at least five minutes. Single-unit signals were amplified 10 times, filtered using a high-pass filter at 30 Hz and a low-pass filter at 10kHz, and further amplified 50 times (Multiclamp 700b, Axon Instruments, USA). The signal was digitized at 16 kHz (CED 1401) and acquired on a computer using Spike 2 7.0 software (Cambridge Electronics Design, Cambridge, UK). Bursts were detected using characteristic parameters of pyramidal neurons (Vitrac et al., 2014) (maximum interval to start a burst: 40 ms, maximum interval to end a burst: 10 ms, minimum interval between burst: 20 ms, minimum duration of a burst: 5 ms and minimum number of spikes in a burst: 2) and analyzed using NeuroExplorer® (Nex Technologies, Colorado Springs, USA).

For all recorded cells, the following electrophysiological parameters were analyzed: basal firing rate, bursting rate, and the percentage of spikes in bursts.

## Histology

Electrode placements were verified via electrophoretic ejection of Chicago Sky Blue dye (Sigma) at the recording site. Rats were euthanized with a lethal dose of pentobarbital (182.2 mg/kg, IP), and brains were removed. The tissue was fixed in 8% paraformaldehyde for at least 48 h and transferred to a 25% sucrose solution for cryoprotection. Once saturated, the brains were frozen and sliced coronally at 60  $\mu$ m thick using a cryostat (Thermo Scientific Cryostat CryoStar NX70) and mounted onto gelatin-coated slides. Tissue was stained with a combination of neutral red and cresyl violet. Only rats with verified electrode placements for both recording and infusion sites were included in the data analysis.

## Statistical Analysis

The sample size and animal numbers were determined by power analysis of pre-existing data (Belujon and Grace, 2014; Chang and Grace, 2014; Belujon et al., 2016; Salin et al., 2021).

Data were analyzed with the statistical program GraphPad Prism (Version 8.0.1; GraphPad Prism, RRID:SCR\_002798).

All the results are expressed as the mean  $\pm$  SEM. A Grubb's test was used to identify statistical outliers' animal. There was no outlier in this study. The level of probability ( $P$ ), for determining groups' differences, was set at  $P < 0.05$ . We followed significant main effects and interactions ( $P < 0.05$ ) with post hoc tests (Sidak's multicomparison test) which were conducted only if the  $F$  values in the analyses achieved the appropriate level of statistical significance and the statistical measures of homogeneity of variance were not significant.

For active/inactive responses in yoked saline animals, a two-way repeated measure (RM) ANOVA was performed with session as the within-subject factor and number of nose-pokes as the between-subject factor.

Since corresponding data for cocaine animals did not pass normality test, a mixed-effects model was used. For incubation of cocaine craving, a paired Mann Whitney's test was performed.

Electrophysiological data were analyzed using either a two-way ANOVA with time (WD1-3 and WD26-45) and drug (saline and cocaine) as factors followed by Sidak's post-hoc when comparing more than two experimental conditions. When comparing two groups, first a Kolmogorov-Smirnov normality test was used. If data passed the normality test, a unpaired t-test was used; otherwise, an unpaired Mann Whitney's test was used.

Complete statistical analyses and exact P values are reported in Table S1.

## Results

### Cocaine self-administration and cocaine seeking behavior after 1 and 30 days of withdrawal

All rats were allowed to self-administer cocaine for 10 sessions under an FR1 schedule of reinforcement. During the 10 sessions of self-administration, cocaine rats had more active than inactive nose-pokes (Figure 1B; n=53), whereas there were no differences in yoked saline rats (Figure 1C; n=21). The number of injections was stable across the self-administration sessions in cocaine rats (Figure 1D). As previously described (Grimm et al., 2001; Chauvet et al., 2012), cocaine seeking (measured in a 1h-extinction session) was significantly higher after 30 days of withdrawal compared to 1 day of withdrawal (Figure 1E; n=6).

### Abstinence from cocaine intake induces changes in dopaminergic activity in the VTA after 30 days of withdrawal, but not 1 day of withdrawal

We have previously shown a significant persistent decrease in the dopaminergic population activity recorded in the VTA after 7 and 30 days of abstinence from extended cocaine intake (25 days) (Salin et al., 2021). Here, we investigated potential changes in DA VTA activity after a shorter 10-day period of cocaine self-administration, in the early phase of abstinence (withdrawal days 1-3) and after protracted abstinence (withdrawal days 26-45). An example of the location of the recording electrode at the end of a recording is presented in Figure 2A. Population activity (Figure 2B), firing rate (Figure 2C) and percentage of spikes firing in bursts (Figure 2D) were analyzed for VTA DA neurons in saline/naive and cocaine rats after 1-3 and 26-45 days of abstinence. Exposure to cocaine induced a significant decrease in population activity of VTA DA neurons during long-term but not short-term abstinence, compared to saline/naive rats (Figure 2B). Post-hoc analysis revealed a significant difference between saline/naive WD1-3 and cocaine WD26-45, saline/naive WD26-45 and cocaine WD26-45 and cocaine WD1-3 and cocaine WD26-45. There was no change in the firing rate (Figure 2C), but a significant drug effect was observed for the percentage of spike firing in bursts (Figure 2D) of DA neurons between groups.

### Abstinence from cocaine intake induces changes in neuronal activity in the BLA after 26-45 days of withdrawal, but not 1-3 days of withdrawal

Previous studies have shown that chronic cocaine intake increases the spontaneous firing rate of BLA neurons after 15 days of abstinence (Munshi et al., 2019) and that the BLA is part of the regulating circuit that decreases tonic DA (Chang and Grace, 2014). Here, we investigated the firing rate and the percentage of spikes firing in burst of BLA neurons in saline and cocaine rats after 1-3 and 26-45 days of abstinence (Figure 3). An example of the location of the recording electrode is presented in Figure 3A. An example of spike doublet and triplet (Rainnie et al., 1993) recorded from a putative projection neuron (Washburn and Moises, 1992; Bienvenu et al., 2012) in the BLA and an example of a spike shape (averaged over 5 minutes) are presented in Figure 3B. We found a significant interaction between time and drug effects for the firing rate (Figure 3C, D) and the percentage of spikes in burst (Figure 3 C, D) in cocaine WD26-45 rats. Post-hoc analysis revealed a significant difference between saline WD26-45 and cocaine WD 30, saline WD1-3 and cocaine WD26-45 and cocaine WD1-3 and cocaine WD26-45 for the firing rate and a significant difference between cocaine WD1-3 and cocaine WD26-45 for the percentage of spikes in burst.

### Attenuation of BLA activity restores DA neuron activity after long-term abstinence from

## cocaine

The DA system is regulated by an inhibitory circuit including the BLA (Belujon et al., 2016; Belujon and Grace, 2017), and amygdala-driven attenuation of DA neuron activity has been described after withdrawal from acute amphetamine (Belujon et al., 2016). Thus, we then tested whether DA neuron population activity could be restored by attenuating BLA activity by local infusion of the sodium channel blocker TTX (or vehicle), immediately before VTA recordings. A representative example of placement of the infusion cannula is presented figure 4A, and the location of the vehicle and TTX infusion cannula is presented figure 4B. As expected, cocaine rats infused with vehicle (VEH) after 26-45 days abstinence had fewer spontaneously active DA neurons, in comparison to after inactivation of BLA activity with TTX (Figure 4C left). No differences in the mean firing rate (figure 4C middle) or the percentage of spikes in a burst (figure 4C right) were observed.

### **Blockade of ventral pallidum glutamatergic inputs restored DA neuron activity after long-term-abstinence from chronic cocaine.**

The ventral pallidum (VP) attenuates VTA DA neuron population activity (Floresco et al., 2001), receives glutamatergic inputs from the BLA (Maslowski-Cobuzzi and Napier, 1994), and blocking glutamatergic inputs in the VP using kynurenic acid has been shown to restore DA population activity in the CMS model of depression (Chang and Grace, 2014). We used kynurenic acid, a broad-spectrum glutamate receptor antagonist (Floresco et al., 2001; Chang and Grace, 2014) to block glutamatergic afferents from the BLA to the VP. A representative example of placement of the infusion cannula is presented figure 5A, and the location of the vehicle and kynurenic acid (KYN) infusion cannula is presented figure 5B. Cocaine rats infused with vehicle (VEH) after 26-45 days abstinence had fewer spontaneously active DA neurons, in comparison to when VP glutamatergic inputs were blocked by kynurenic acid (Figure 5C left). No differences in the mean firing rate (figure 5C middle) or the percentage of spikes in a burst (figure 5C right) were observed.

## Discussion

In this study, we found a significant decrease of VTA DA population activity after long term, but not short term, abstinence from chronic cocaine self-administration, a pattern paralleling the phenomenon of incubation of craving (Grimm et al., 2001). We also found a significant increase of BLA activity after long but not short-term, abstinence. Finally, we show that the decrease in VTA DA activity depends on the BLA-VP pathway.

We have previously shown that extended exposure to cocaine (25 days), followed by abstinence (7 and 30 days) persistently decreases DA activity in the VTA (Salin et al., 2021). Here, we extend these findings using a shorter 10 day-self-administration protocol, showing a decrease in DA population activity after 26-45 days but not 1-3 days of abstinence. We found no changes of DA population activity during acute withdrawal, suggesting no changes in tonic DA release in VTA target structures (Floresco et al., 2003) at this time point. This is consistent with some studies using microdialysis that showed no changes in baseline DA during acute withdrawal (Calipari et al., 2014; Cameron et al., 2016), but it is inconsistent with other work (Hurd et al., 1989; Weiss et al., 1992) demonstrating reduced basal DA levels during acute withdrawal. There are multiple differences between studies that can account for these discrepancies, including the use of slice versus *in vivo* preparation, the type of self-administration paradigm (length of the self-administration period for example), and the time of microdialysis measurements relative to the last self-administration sessions (12h, 24h or up to 72h after the last self-administration session). In our study, DA neurons' activity was recorded from 18h up to 72 h after the last session, suggesting that potential decreases in population activity and subsequent decreased DA release might normalize rapidly following discontinuation of cocaine self-administration. Another explanation could be that tonic DA decreases independently of population activity (via changes in presynaptic DA release or increased reuptake). Although there are discrepancies in changes of DA level or DA activity during acute withdrawal, a decrease in DA activity has been consistently described in the literature following long-term withdrawal (Salin et al., 2021; Shen et al., 2007). This decrease could participate in the dysphoria described after protracted cocaine abstinence (Haake et al., 2019).

Previous work has shown that activation of the BLA potently decreases the number of DA neuron firing (Chang and Grace, 2014). Increased activity in the BLA has been previously described after extended withdrawal from chronic cocaine (Munshi et al., 2019), which is consistent with the increased firing rate and percentage of spikes in bursts after long-term abstinence in our study. However, Munshi et al did not study BLA activity after acute withdrawal. Here, we show no changes in the firing rate or the percentage of spikes firing in burst after 1-3 days of withdrawal. These changes of firing rate in the BLA across the abstinence period could be attributed to multiples factors, such as changes in intrinsic excitability, in the expression of NMDA or AMPA receptors, or changes in different inputs to pyramidal neurons. Changes in membrane properties have been described in several pathological conditions such as drug addiction (Kourrich et al., 2015). Numerous studies have described increased excitability of BLA neurons after chronic stress (Rosenkranz et al., 2010; Rau et al., 2015). Since chronic stress and drug of abuse share common substrates (Belujon and Grace, 2011; Munshi et al., 2019), the increased firing rate and bursting activity of BLA neurons after long abstinence could be due to increased neuronal excitability. Another factor that might contribute to increased firing is changes in the expression of NMDA or AMPA receptors in the BLA. Indeed, previous work has shown an increased in the expression of GLUR1 and GLUR2 subunits as well as a decrease in the expression of NR2B subunits in the BLA during cocaine abstinence (Lu et al., 2005). Interneurons in the BLA tightly regulate excitability of principal neurons (Ehrlich et al., 2009) by targeting their perisomatic region (Bienvenu et al., 2012) and blocking activity in principal neurons (Woodruff and Sah, 2007). Since activity of BLA principal neurons is regulated by local interneurons, dysregulation of interneurons' activity in the BLA after chronic cocaine could increase spontaneous activity of principal neurons. Although increased activity of parvalbumin (PV) interneurons has been described in the central amygdala after chronic opiates withdrawal (Wang et al., 2016), there is a lack of information concerning PV interneurons in the BLA after psychostimulant withdrawal. Moreover, BLA activity can be potently modulated by the prefrontal cortex through inhibitory control via reciprocal connections (Rosenkranz and Grace, 2002). Changes in activity in the medial prefrontal cortex has been observed in abstinent cocaine abusers (Bolla et al., 2004) which could lead to pathological activation of the amygdala. Therefore, after protracted abstinence of chronic cocaine hyperactivity of the BLA could underlie the reduced DA VTA population activity observed in the present study.

Chronic stress has been shown to increase BLA activity (Munshi et al., 2019) and decrease DA population activity in the VTA (Chang and Grace, 2014). Inactivation of the BLA in a model of chronic stress has also been shown to increase DA population activity (Chang and Grace, 2014). In our model, we tested the effect of BLA inactivation on DA population activity after long-term abstinence. In agreement with the stress literature (Chang and Grace, 2014), BLA inactivation increased dopaminergic activity in cocaine rats after long-term withdrawal. These results support the existence of common substrates between addiction and stress-related emotional disorders (Belujon and Grace, 2011; Polter and Kauer, 2014), and emphasize that normalization of BLA activity may be involved in reducing abstinence-related emotional symptoms.

The BLA does not project directly to the VTA but has strong projections to the VP (Maslowski-Cobuzzi and Napier, 1994) and the BLA-VP-VTA has been shown to exert potent action on DA neuron activity states (Chang and Grace, 2014). In our model, blocking glutamatergic afferents in the VP after long-term abstinence restores DA activity suggesting that the BLA-VP pathway is involved in the decreased DA activity after protracted abstinence. Mahler et al. have shown that GABAergic neurons from the rostral part of the VP (RVP) projecting to the VTA, but not the caudal part, is activated during cue-induced reinstatement and that inactivation of the RVP-VTA pathway reduces cocaine seeking behavior after long-term abstinence (Mahler et al., 2014). In our study, glutamatergic afferents were blocked in the RVP, suggesting that increased activity of the BLA-RVP pathway leading to hypodopaminergic activity in the VTA after long-term withdrawal might play a critical role in cue-induced seeking. Other regions such as RMTg could be an additional relay structure in restoring DA activity since it receives direct projections from the BLA (Kaufling et al., 2009), and sends direct GABAergic inhibitory afferents to the VTA (Lecca et al., 2012). However, RMTg activation inhibits DA neurons firing rate (Lecca et al., 2012), but firing rate of VTA neurons was not altered during withdrawal in our study.

## Conclusion

Our study sheds new lights on neuroadaptations occurring during incubation of craving, a phenomenon believed to play a role in persistent risks of relapse, which is crucial to improve novel therapeutic strategies. In particular, we demonstrate that after long-term withdrawal the decrease of DA activity in the VTA is associated with hyperactivity of the BLA and involved a circuit comprising the BLA and the VP. Dysfunctions of this circuit could underlie dysphoria, which has been described during acute but also long-term withdrawal from cocaine. Imaging studies focusing on the interactions of regions in this specific network could be used as a marker of vulnerability to dysphoria, craving and relapse.

## References

- Adhikari, A., Lerner, T.N., Finkelstein, J., Pak, S., Jennings, J.H., Davidson, T.J., et al. (2015). Basomedial amygdala mediates top-down control of anxiety and fear. *Nature* *527* : 179–185.
- Belujon, P., and Grace, A.A. (2011). Hippocampus, amygdala, and stress: interacting systems that affect susceptibility to addiction. *Ann N Acad Sci* *1216* : 114–21.
- Belujon, P., and Grace, A.A. (2014). Restoring mood balance in depression: ketamine reverses deficit in dopamine-dependent synaptic plasticity. *Biol Psychiatry* *76* : 927–36.
- Belujon, P., Jakobowski, N.L., Dollish, H.K., and Grace, A.A. (2016). Withdrawal from Acute Amphetamine Induces an Amygdala-Driven Attenuation of Dopamine Neuron Activity: Reversal by Ketamine. *Neuropsychopharmacology* *41* : 619–27.
- Belujon, P., and Grace, A.A. (2017). Dopamine System Dysregulation in Major Depressive Disorders. *Int. J. Neuropsychopharmacol.* *20* : 1036–1046.
- Bienvendu, T.C.M., Busti, D., Magill, P.J., Ferraguti, F., and Capogna, M. (2012). Cell-Type-Specific Recruitment of Amygdala Interneurons to Hippocampal Theta Rhythm and Noxious Stimuli In Vivo. *Neuron* *74*–20 : 1059–1074.
- Bolla, K., Ernst, M., Kiehl, K., Mouratidis, M., Eldreth, D., Contoreggi, C., et al. (2004). Prefrontal cortical dysfunction in abstinent cocaine abusers. *J. Neuropsychiatry Clin. Neurosci.* *16* : 456–464.
- Calipari, E.S., Ferris, M.J., and Jones, S.R. (2014). Extended access of cocaine self-administration results in tolerance to the dopamine-elevating and locomotor-stimulating effects of cocaine. *J. Neurochem.* *128* : 224–232.
- Cameron, C.M., Wightman, R.M., and Carelli, R.M. (2016). One month of cocaine abstinence potentiates rapid dopamine signaling in the nucleus accumbens core. *Neuropharmacology* *111* : 223–230.
- Chang, C., and Grace, A.A. (2014). Amygdala-Ventral Pallidum Pathway Decreases Dopamine Activity After Chronic Mild Stress in Rats. *Biol. Psychiatry* *76* : 223–230.
- Chauvet, C., Goldberg, S.R., Jaber, M., and Solinas, M. (2012). Effects of environmental enrichment on the incubation of cocaine craving. *Neuropharmacology* *63* : 635–41.
- Childress, A.R., Mozley, P.D., McElgin, W., Fitzgerald, J., Reivich, M., and O'Brien, C.P. (1999). Limbic Activation During Cue-Induced Cocaine Craving. *Am. J. Psychiatry* *156* : 11–18.
- Diana, M., Pistis, M., Muntoni, A., and Gessa, G. (1996). Mesolimbic dopaminergic reduction outlasts ethanol withdrawal syndrome: evidence of protracted abstinence. *Neuroscience* *71* : 411–5.
- Ehrlich, I., Humeau, Y., Grenier, F., Cioocchi, S., Herry, C., and Luthi, A. (2009). Amygdala inhibitory circuits and the control of fear memory. *Neuron* *62* : 757–771.
- Farrell, M.R., Ruiz, C.M., Castillo, E., Faget, L., Khanbiji, C., Liu, S., et al. (2019). Ventral pallidum is essential for cocaine relapse after voluntary abstinence in rats. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* *44* : 2174–2185.

- Floresco, S.B., Todd, C.L., and Grace, A.A. (2001). Glutamatergic afferents from the hippocampus to the nucleus accumbens regulate activity of ventral tegmental area dopamine neurons. *J Neurosci* *21* : 4915–22.
- Floresco, S.B., West, A.R., Ash, B., Moore, H., and Grace, A.A. (2003). Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci* *6* : 968–73.
- Garavan, H., Pankiewicz, J., Bloom, A., Cho, J.-K., Sperry, L., Ross, T.J., et al. (2000). Cue-Induced Cocaine Craving: Neuroanatomical Specificity for Drug Users and Drug Stimuli. *Am. J. Psychiatry* *157* : 1789–1798.
- Grimm, J.W., Hope, B.T., Wise, R.A., and Shaham, Y. (2001). Neuroadaptation. Incubation of cocaine craving after withdrawal. *Nature* *412* : 141–2.
- Haake, R.M., West, E.A., Wang, X., and Carelli, R.M. (2019). Drug-induced dysphoria is enhanced following prolonged cocaine abstinence and dynamically tracked by nucleus accumbens neurons. *Addict. Biol.* *24* : 631–640.
- Huijstee, A.N. van, and Mansvelder, H.D. (2015). Glutamatergic synaptic plasticity in the mesocorticolimbic system in addiction. *Front. Cell. Neurosci.* *8* : .
- Hurd, Y.L., Weiss, F., Koob, G.F., And, N.E., and Ungerstedt, U. (1989). Cocaine reinforcement and extracellular dopamine overflow in rat nucleus accumbens: an in vivo microdialysis study. *Brain Res.* *498* : 199–203.
- Janak, P.H., and Tye, K.M. (2015). From circuits to behaviour in the amygdala. *Nature* *517* : 284–292.
- Kauffling, J., Veinante, P., Pawlowski, S.A., Freund-Mercier, M.-J., and Barrot, M. (2009). Afferents to the GABAergic tail of the ventral tegmental area in the rat. *J. Comp. Neurol.* *513* : 597–621.
- Kourrich, S., Calu, D.J., and Bonci, A. (2015). Intrinsic plasticity: an emerging player in addiction. *Nat. Rev. Neurosci.* *16* : 173–184.
- Lecca, S., Melis, M., Luchicchi, A., Muntoni, A.L., and Pistis, M. (2012). Inhibitory inputs from rostromedial tegmental neurons regulate spontaneous activity of midbrain dopamine cells and their responses to drugs of abuse. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* *37* : 1164–1176.
- LeDoux, J.E. (2000). Emotion Circuits in the Brain. *Annu. Rev. Neurosci.* *23* : 155–184.
- Lu, L., Dempsey, J., Shaham, Y., and Hope, B.T. (2005). Differential long-term neuroadaptations of glutamate receptors in the basolateral and central amygdala after withdrawal from cocaine self-administration in rats. *J. Neurochem.* *94* : 161–168.
- Maas, L.C., Lukas, S.E., Kaufman, M.J., Weiss, R.D., Daniels, S.L., Rogers, V.W., et al. (1998). Functional Magnetic Resonance Imaging of Human Brain Activation During Cue-Induced Cocaine Craving. *Am. J. Psychiatry* *155* : 124–126.
- Mahler, S.V., Vazey, E.M., Beckley, J.T., Keistler, C.R., McGlinchey, E.M., Kauffling, J., et al. (2014). Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. *Nat Neurosci* *17* : 577–85.
- Maslowski-Cobuzzi, R.J., and Napier, T.C. (1994). Activation of dopaminergic neurons modulates ventral pallidal responses evoked by amygdala stimulation. *Neuroscience* *62* : 1103–19.
- Meil, W.M., and See, R.E. (1997). Lesions of the basolateral amygdala abolish the ability of drug associated cues to reinstate responding during withdrawal from self-administered cocaine. *Behav Brain Res* *87* : 139–48.
- Munshi, S., Rosenkranz, J.A., Caccamise, A., Wolf, M.E., Corbett, C.M., and Loweth, J.A. (2019). Cocaine and chronic stress exposure produce an additive increase in neuronal activity in the basolateral amygdala. *Addict. Biol.* e12848.

- Murray, E.A. (2007). The amygdala, reward and emotion. *Trends Cogn. Sci.* *11* : 489–497.
- Polter, A.M., and Kauer, J.A. (2014). Stress and VTA synapses: implications for addiction and depression. *Eur. J. Neurosci.* *39* : 1179–1188.
- Rainnie, D.G., Asprodini, E.K., and Shinnick-Gallagher, P. (1993). Intracellular recordings from morphologically identified neurons of the basolateral amygdala. *J. Neurophysiol.* *69* : 1350–1362.
- Rau, A.R., Chappell, A.M., Butler, T.R., Ariwodola, O.J., and Weiner, J.L. (2015). Increased Basolateral Amygdala Pyramidal Cell Excitability May Contribute to the Anxiogenic Phenotype Induced by Chronic Early-Life Stress. *J. Neurosci.* *35* : 9730–9740.
- Rosenkranz, J.A., and Grace, A.A. (2002). Cellular mechanisms of infralimbic and prelimbic prefrontal cortical inhibition and dopaminergic modulation of basolateral amygdala neurons in vivo. *J. Neurosci. Off. J. Soc. Neurosci.* *22* : 324–337.
- Rosenkranz, J.A., Venheim, E.R., and Padival, M. (2010). Chronic stress causes amygdala hyperexcitability in rodents. *Biol. Psychiatry* *67* : 1128–1136.
- Salin, A., Lardeux, V., Solinas, M., and Belujon, P. (2021). Protracted Abstinence From Extended Cocaine Self-Administration Is Associated With Hypodopaminergic Activity in the VTA but Not in the SNc. *Int. J. Neuropsychopharmacol.* *24* : 499–504.
- Shen, R.-Y., Choong, K.-C., and Thompson, A.C. (2007). Long-Term Reduction in Ventral Tegmental Area Dopamine Neuron Population Activity Following Repeated Stimulant or Ethanol Treatment. *Biol. Psychiatry* *61* : 93–100.
- Smith, K.S., Tindell, A.J., Aldridge, J.W., and Berridge, K.C. (2009). Ventral pallidum roles in reward and motivation. *Behav. Brain Res.* *196* : 155–167.
- Swanson, L.W. (2018). Brain maps 4.0—Structure of the rat brain: An open access atlas with global nervous system nomenclature ontology and flatmaps. *J. Comp. Neurol.* *526* : 935–943.
- Vitrac, C., Peron, S., Frappe, I., Fernagut, P.-O., Jaber, M., Gaillard, A., et al. (2014). Dopamine control of pyramidal neuron activity in the primary motor cortex via D2 receptors. *Front. Neural Circuits* *8* :.
- Wang, L., Shen, M., Jiang, C., Ma, L., and Wang, F. (2016). Parvalbumin Interneurons of Central Amygdala Regulate the Negative Affective States and the Expression of Corticotrophin-Releasing Hormone During Morphine Withdrawal. *Int. J. Neuropsychopharmacol.* *19* : pyw060.
- Washburn, M.S., and Moises, H.C. (1992). Electrophysiological and morphological properties of rat basolateral amygdaloid neurons in vitro. *J. Neurosci. Off. J. Soc. Neurosci.* *12* : 4066–4079.
- Wassum, K.M., and Izquierdo, A. (2015). The basolateral amygdala in reward learning and addiction. *Neurosci. Biobehav. Rev.* *57* : 271–283.
- Weiss, F., Markou, A., Lorang, M.T., and Koob, G.F. (1992). Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration. *Brain Res* *593* : 314–8.
- Wise, R.A., and Hoffman, D.C. (1992). Localization of drug reward mechanisms by intracranial injections. *Synapse. N. Y. N* *10* : 247–263.
- Wise, R.A. (2008). Dopamine and reward: The anhedonia hypothesis 30 years on. *Neurotox. Res.* *14* : 169–183.
- Woodruff, A.R., and Sah, P. (2007). Inhibition and synchronization of basal amygdala principal neuron spiking by parvalbumin-positive interneurons. *J. Neurophysiol.* *98* : 2956–2961.

## Figure legends

## Figure 1.

**Self-administration and relapse test.** Experimental design timeline (A). Number of active (black) and inactive (white) nose-pokes during the 10 sessions in cocaine rats (B) and in yoked saline rats (C). Number of cocaine injections (0.75mg/kg/infusion) during the ten self-administration sessions (D). Number of active responses during cocaine-seeking over 1 h-session 24h (WD1) and 30 days (WD30) after the last self-administration session (E).

Data are mean  $\pm$  SEM; \* $p < 0.05$ ; cocaine rats:  $n = 53$ , yoked saline rats:  $n = 21$ , cocaine seeking:  $n = 6$ .

## Figure 2.

**Dopaminergic activity in the ventral tegmental area after short- (WD1) and long- (WD30) term withdrawal from chronic cocaine and yoked saline.** Histological slice showing a Chicago sky blue deposit dot in the VTA (A). Mean number of spontaneously active DA neurons recorded per electrode track (B), number of spikes per second (firing rate) and percentage of action potential firing in bursts (D) in saline/naive and cocaine rats after 18h-72h (WD 1-3) and saline/naive and cocaine rats after 26-45 days after the last self-administration session (WD 26-45).

Data are mean  $\pm$  SEM; \* $p < 0.05$ ; saline/naive WD1-3: 9 rats (5 saline, 4 naive), 65 neurons; cocaine WD1-3: 5 rats, 60 neurons; saline/naive WD26-45: 9 rats (6 Saline, 3 naive), 65 neurons; cocaine WD26-45: 6 rats, 30 neurons. Coronal brain sections adapted from (Swanson, 2018).

## Figure 3.

**Activity of neurons recorded from the basolateral amygdala after short- (WD1-3) and long- (WD26-45) term withdrawal from chronic cocaine and yoked saline .** Histological slice showing a Chicago sky blue deposit dot in the BLA (A). Representative electrophysiological trace of a BLA neurons showing the presence of triplet and doublet of spikes (black arrows). Inset represents the shape of action potentials (averaged over 5 minutes), with the action potential duration is measured from peak to trough (dashed line) (B). Two-minute representative electrophysiological trace of a BLA neurons recorded from (top to bottom) : saline WD1-3, cocaine WD1-3, saline WD26-45 and cocaine WD26-45 rats (C). Number of action potentials per second (firing rate; top) and percentage of action potentials firing in burst (bottom). On the left, individual data points and mean  $\pm$  SEM; on the right, mean  $\pm$  SEM and statistics (D). \* $p < 0.05$ . saline WD1-3: 35 neurons; cocaine WD1-3: 30 neurons; saline WD26-45: 36 neurons; cocaine WD26-45: 56 neurons. Coronal brain sections adapted from (Swanson, 2018).

## Figure 4.

**Effect of inactivating the BLA with TTX on VTA DA neuron activity.** Histological slice showing track of the guide cannula in the BLA (A). Coronal brain section adapted from (Swanson, 2018) (VEH: black dots; TTX: white and black dots) (B). Number of spontaneously active DA neurons recorded per electrode track (C, left), number of spikes per second (firing rate; C, middle) and percentage of action potential firing in bursts (C, right) in cocaine WD26-45 rats after VEH or TTX infusion in the BLA. Data are mean  $\pm$  SEM; \* $p < 0.05$ ; cocaine WD26-45 VEH: 7 rats, 32 neurons; cocaine WD26-45 TTX: 6 rats, 50 neurons. Coronal brain sections adapted from (Swanson, 2018).

## Figure 5.

**Effect of blocking glutamatergic afferents with kynurenic acid in the ventral pallidum on VTA DA neuron activity.** Histological slice showing track of the guide cannula in the VP (A). Coronal brain section adapted from (Swanson, 2018) (VEH: black dots; KYN: white and black dots) (B). Number of spontaneously active DA neurons recorded per electrode track (C, left), number of spikes per second (firing rate; C, middle) and percentage of action potential firing in bursts (C, right) in cocaine WD30 rats after VEH or KYN infusion in the VP. Data are mean  $\pm$  SEM; \* $p < 0.05$ ; cocaine WD26-45 VEH: 5 rats, 27 neurons; cocaine WD26-45 KYN: 5 rats, 49 neurons. Coronal brain sections adapted from (Swanson, 2018).



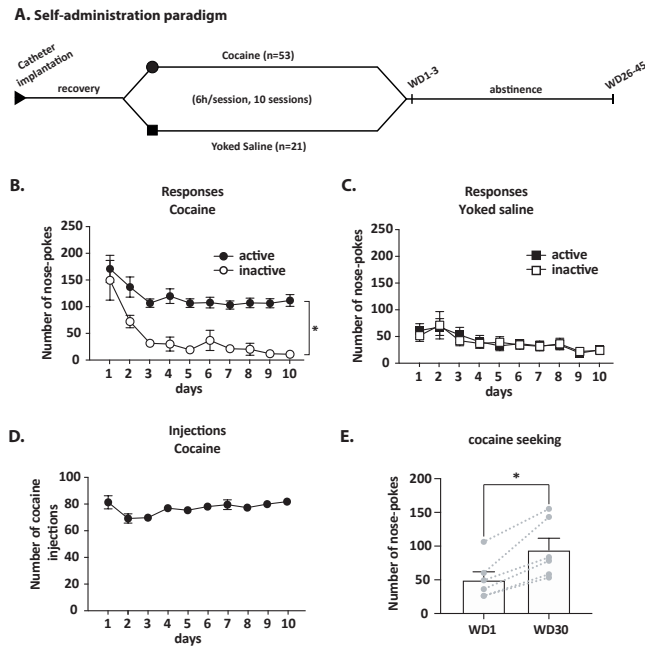


Figure 1

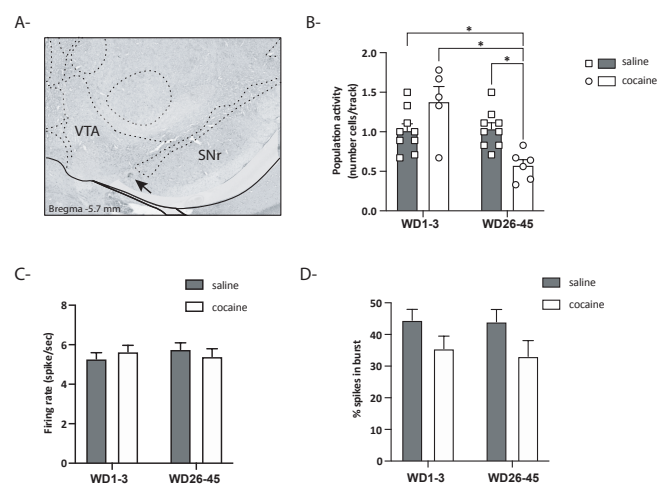


Figure 2

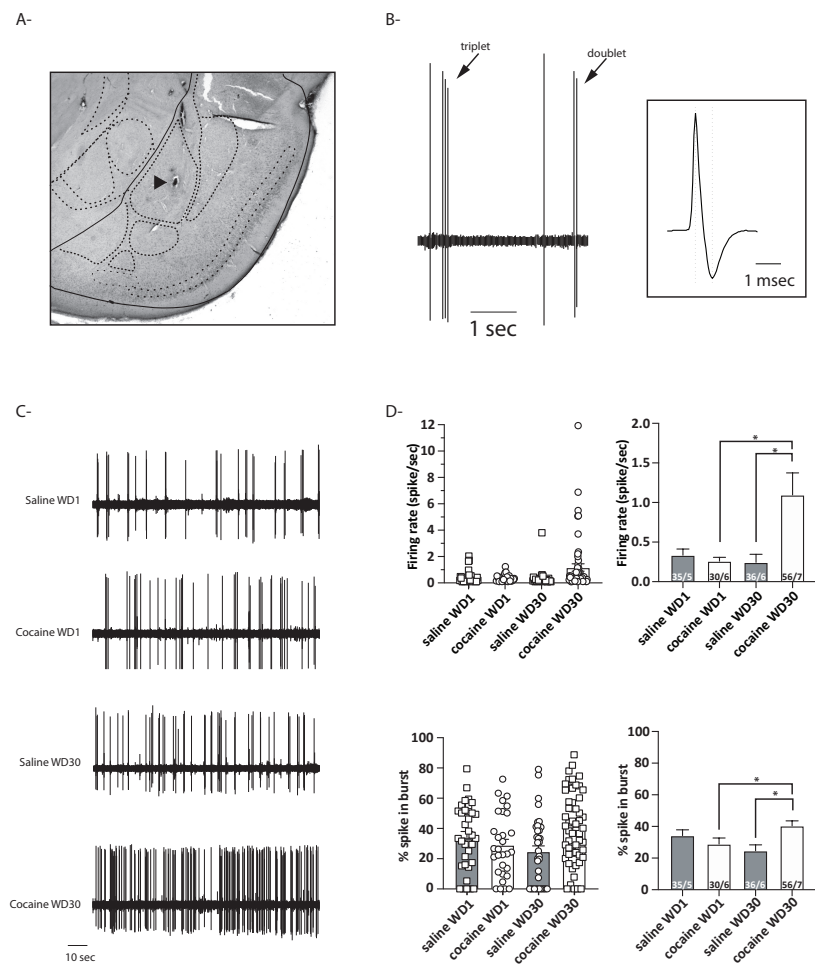


figure 3

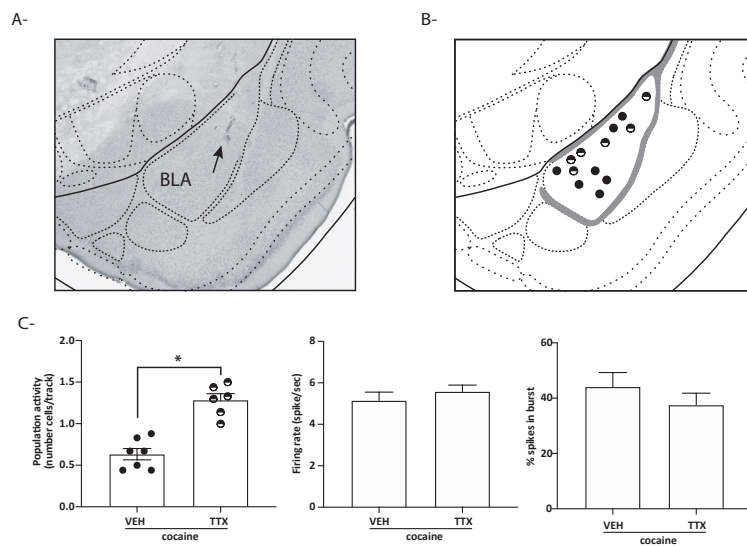


Figure 4

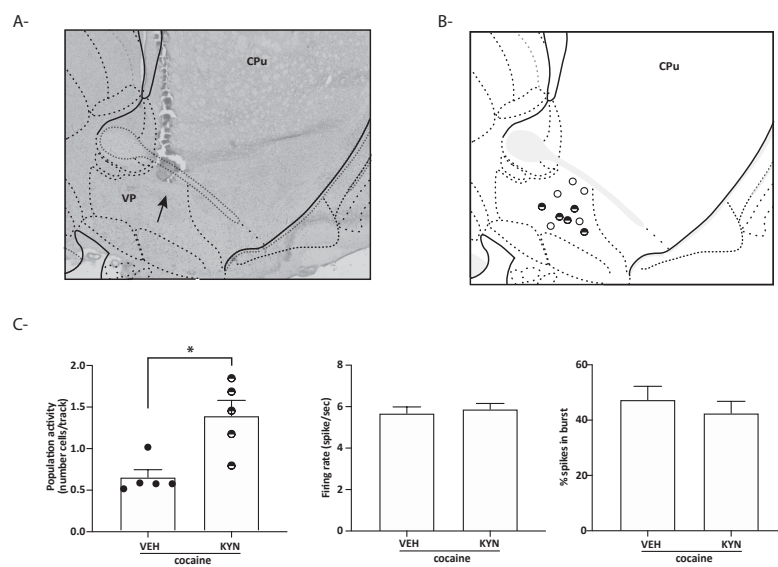
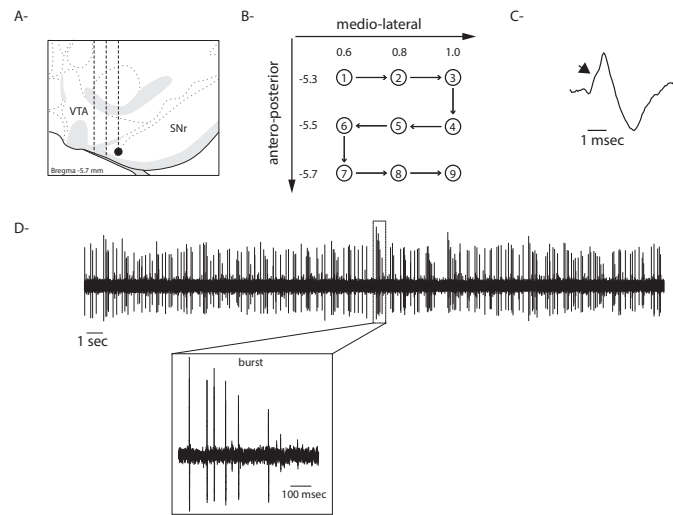


Figure 5



**Figure S1. Electrophysiological identification of dopaminergic neurons.**

(A) Coronal sections of the brain atlas adapted from (Swanson, 2018) showing representative location of three recording tracks in the VTA (dashed line) and location of the deposit of the Chicago sky blue dye (black dot) B) Schematic representing the pattern of the cell/track sampling of the VTA. The population activity of dopamine (DA) neurons is determined by counting the number of spontaneously firing DA neurons encountered while making 5 to 9 vertical tracks separated by 200  $\mu\text{m}$ . C) Waveform of an extracellular action potential presented as an example. As previously described (Grace and Bunney, 1983), neurons display a biphasic (positive-negative) action potential, typically with a "notch" in the rising phase corresponding to the calcium-dependent initial segment spike (arrow) and a prominent negative component, and with a total duration  $>2.2$  ms overall. The duration from the spike initiation to the maximal negative phase of the action potential was  $\geq 1.1$  ms for all recorded neurons. D) Representative example of a recording of a putative DA neuron; the insert represents a close-up of a burst of action potentials.

Grace, A.A., and Bunney, B.S. (1983). Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--1. Identification and characterization. *Neuroscience* 10: 301–15.

Swanson, L.W. (2018). Brain maps 4.0—Structure of the rat brain: An open access atlas with global nervous system nomenclature ontology and flatmaps. *J. Comp. Neurol.* 526: 935–943.

Figure	Factor name	F value	P value	Statistical test
Figure 1B	Session Nose-pokes Interaction	$F_{9,928} = 12.02$ $F_{1,104} = 55.39$ $F_{9,928} = 1.572$	<b>*p&lt;0.0001</b> <b>*p&lt;0.0001</b> p=0.1189	Mixed effect analysis
Figure 1C	Session Nose-pokes Interaction	$F_{9,357} = 8.239$ $F_{1,40} = 0.007687$ $F_{9,357} = 0.2847$	<b>*p= 0.0010</b> p= 0.9306 p= 0.9787	Mixed effect analysis
Figure 1D	Session	$F_{9,41} = 2.692$	<b>*p=0.0365</b>	RM One- way ANOVA
Figure 1E	Withdrawal day		<b>*p=0.0312</b>	Wilcoxon test
Figure 2B	Withdrawal day Drug Interaction	$F_{1,25} = 13.25$ $F_{1,25} = 0.1987$ $F_{1,25} = 14.88$	<b>*p=0.0012</b> p=0.6596 <b>*p=0.007</b>	Two-way ANOVA
Figure 2C	Withdrawal day) Drug Interaction	$F_{1,215} = 0.111$ $F_{1,215} = 5.983e-030$ $F_{1,215} = 0.9939$	p= 0.7393 p>0 .9999 p= 0.3199	Two-way ANOVA
Figure 2D	Withdrawal day Drug Interaction	$F_{1,215} = 0.1146$ $F_{1,215} = 5.561$ $F_{1,215} = 0.05059$	p= 0.7353 <b>*p= 0.0193</b> p= 0.8223	Two-way ANOVA
Figure 3D, top	Withdrawal day Drug Interaction	$F_{1,153} = 3.048$ $F_{1,153} = 3.313$ $F_{1,153} = 4.691$	p=0.0828 p=0.0707 <b>*p= 0.0319</b>	Two-way ANOVA
Figure 3D, bottom	Withdrawal day Drug Interaction	$F_{1,153} = 0.06625$ $F_{1,153} = 2.033$ $F_{1,153} = 8.296$	p=0.7972 p=0.156 <b>*p= 0.0045</b>	Two-way ANOVA
Figure 4C left	infusion		<b>*p=0.0012</b>	Mann- Whitney test
Figure 4C center	infusion		p= 0.3473	Unpaired t test
Figure 4C right	infusion		p= 0.2132	Unpaired t test
Figure 5C left	infusion		<b>*p=0.0159</b>	Mann- Whitney test
Figure 5C center	infusion		p= 0.6488	Unpaired t test
Figure 5C right	infusion		p= 0.4814	Mann- Whitney test

**Supplementary table 1.** Statistical analysis (GraphPad Prism)