Published in final edited form as:

Neuroscience. 2012 April 5; 207: 182–197. doi:10.1016/j.neuroscience.2012.01.026.

Ventral tegmental area neurons are either excited or inhibited by cocaine's actions in the peripheral nervous system

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Abstract

Cocaine's multiple pharmacological substrates are ubiquitously present in the peripheral and central nervous system. Thus, upon its administration, cocaine acts in the periphery before directly acting in the brain. We determined whether cocaine alters ventral tegmental area (VTA) neuronal activity via peripheral actions, and whether this precedes its central actions. In urethane-anesthetized rats, we recorded VTA neurons responses to intravenous injections of two cocaine analogs: cocaine-hydrochloride (HCl, 0.25 mg/kg) that readily cross the blood-brain barrier (BBB) and cocaine-methiodide (MI, 0.33 mg/kg) that does not cross the BBB. Both cocaine analogs produced sustained changes in discharge rates that began 5s after the initiation of a 10s drug infusion. Within the first 90s post-injection the magnitudes of neuronal responsive of both cocaine analogs were comparable, but later in time the effects of cocaine-HCl were stronger and persisted longer than those of cocaine-MI. The proportion of neurons responsive to cocaine-HCl was twice to that of cocaine-MI (74% and 35% respectively). Both analogs also differed in the response onsets. Cocaine-MI rarely evoked responses after 1 min whereas cocaine-HCl continued to evoke responses within 3 min post-injection. VTA neurons were either excited or inhibited by both cocaine analogs. Most units responsive to cocaine-MI, regardless of excitation or inhibition, had electrophysiological characteristics of putative DA neurons. Units inhibited by cocaine-HCl also had characteristic of DA neurons whereas excited neurons had widely varying action potential durations and discharge rates. Cocaine-MI and cocaine-HCl each produced changes in VTA neuron activity under full DA receptor blockade. However, the duration of inhibition was shortened, the number of excitations increased, and they occurred with an earlier onset during DA receptor blockade. These findings indicate that cocaine acts peripherally with a short latency and alters the activity of VTA neurons prior to its well-known direct actions in the brain.

Keywords

cocaine methiodide; peripheral nervous system; cocaine; ventral tegmental area; single-unit recordings

The rewarding properties of cocaine are related to its ability to block the dopamine transporter (DAT)(Ritz *et al.*, 1987; Thomsen *et al.*, 2009), thus increasing dopamine (DA) concentrations in cortico-limbic brain areas (Di Chiara and Imperato, 1988; Pettit and

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Justice, 1989). Cocaine-induced DA increase at terminals depends on DA neurons impulse activity (Nomikos *et al.*, 1990; Sombers *et al.*, 2009); consequently, cocaine regulation of DA neuron activity is as essential as its action on the terminal sites. The DA innervation to cortico-limbic brain areas arises primarily from neurons of ventral tegmental area (VTA) (Ungerstedt, 1971; Lindvall and Bjorklund, 1974; Swanson, 1982). VTA DA neurons are partially inhibited by cocaine (Einhorn *et al.*, 1988). This partial inhibition has been regarded as the major effect of cocaine on VTA DA neurons. However, activation of VTA DA neurons by cocaine has also been reported (Einhorn *et al.*, 1988; Zhou *et al.*, 2005; Brown and Kiyatkin, 2008; Steffensen *et al.*, 2008).

In addition to its ability to inhibit the DAT, cocaine acts on other pharmacological substrates including the norepinephrine and serotonin transporters, voltage gated Na⁺ and K⁺ channels, and Ca²⁺-activated K⁺ channels (Reith *et al.*, 1986; Crumb and Clarkson, 1990; Ritz *et al.*, 1990; Zhang *et al.*, 2002; Premkumar, 2005; Kobayashi *et al.*, 2007). The ubiquitous localization of cocaine's pharmacological targets throughout the brain and body provides many pathways through which the activity of VTA DA neurons can be affected. For instance, in addition to local actions on VTA neurons, cocaine can affect the activity VTA neurons before it enters the brain by evoking an interoceptive sensory signal that through the nucleus of the solitary tract or the parabrachial nucleus can reach the VTA (Geisler and Zahm, 2005; Geerling and Loewy, 2006; Mejias-Aponte *et al.*, 2009; Coizet *et al.*, 2010). Previous studies have established that cocaine's peripheral action can be sensed by the cortex as well as the nucleus accumbens (Brown and Kiyatkin, 2006; Kiyatkin and Brown, 2006, 2007; Kiyatkin and Smirnov, 2010). In these studies cocaine-methiodide (MI), a cocaine analog unable to cross the BBB, produced similar responses to cocaine-hydrochloride (HCl) which readily crosses the BBB. In the present study, we investigated the effect of cocaine's interoceptive actions on the neural activity of VTA neurons by contrasting the changes in discharge rate of VTA neurons between cocaine-HCl and cocaine-MI. We also examined to what extent VTA neuronal effects to cocaine depend upon DA transmission by testing the effects of the drugs during full DA receptor blockade induced by a mixture of D1 and D2 selective DA receptor blockers.

Most previous studies of the effects of cocaine on VTA neurons were focused exclusively on putative DA neurons indirectly identified based on specific electrophysiological features (Einhorn *et al.*, 1988; Pitts and Marwah, 1988; Hinerth *et al.*, 2000; Marinelli and White, 2000; Marinelli *et al.*, 2003). In contrast, we tested every neuron within the VTA boundaries. This allowed us to evaluate what the VTA cell population response to cocaine is. The VTA cells were then analyzed on the type of responses evoked by cocaine. Lastly, we described the electrophysiological characteristic features of the responsive neurons. We chose this approach rather than pre-selection of neurons for two main reasons: (1) the lack of a distinguishable selection DA and non-DA neurons (Ungless *et al.*, 2004; Margolis *et al.*, 2006; Luo *et al.*, 2008), and (2) the heterogeneity of VTA cell types such as the glutamate neurons (Yamaguchi *et al.*, 2007; Dobi *et al.*, 2010; Yamaguchi *et al.*, 2011) that have not been characterized by electrophysiological features.

Experimental Procedures

Subjects

Forty-eight male Long-Evans rats (Charles-River, Raleigh, N.C., 360 – 500 g) were used for these experiments. They were housed in pairs until the day of surgery and individually thereafter. All subjects were maintained on a 12-hour light-dark cycle with free access to food and water. The experimental procedures followed the “Principles of Laboratory Animal Care” published by the National Institutes of Health (NIH publication 86-23, 1996), and

were approved by the Animal Care and Use Committee of the NIDA Intramural Research Program.

Intravenous catheterization and placement of the reference electrode

Each rat was anesthetized with a mix of sodium pentobarbital and chloride hydrate (32.5 and 145 mg/kg, i.p., respectively). The rat was mounted in a stereotaxic frame. The skull was exposed and two anchor screws were threaded in the frontal bone. Another screw was placed in the right parietal bone to serve as a reference for single-unit recordings, and to secure an intravenous (i.v.) catheter. The screws in the frontal bone and the base of the screw in the parietal bone were covered with dental acrylic forming an inverted L-shape, keeping bregma and lambda and the central suture visible for taking stereotaxic coordinates on the day of electrophysiological recordings.

For drug administration, an i.v. catheter was placed in the external right jugular vein via a small incision adjacent to the neck midline. The jugular vein was externalized and an i.v. silastic catheter (inner diameter 0.51 mm, outer diameter 0.94 mm; Dow Corning, Midland, MI) was inserted into the vein and secured with a silk suture. The catheter was routed subcutaneously around the back of the neck and externalized at the head's surgical area. The end of the catheter was slipped over a bent and blunted 23-gauge syringe hub, which was secured next to the temporal lobe screw with dental acrylic. To keep the catheter patent, 0.2 ml of a 0.9% saline solution was flushed through the catheter every day following surgery.

Surgeries were performed 3–14 days before electrophysiological recordings under urethane (1) to eliminate the pain or trauma during catheter implantation; (2) to allow more time for recordings; and (3) to match the experimental protocol of previous experiments in awake rats conducted in our lab.

VTA single-unit recording

On the day of electrophysiological recordings, each rat was anesthetized with urethane (1.4 g/kg, i.p.) and mounted in a stereotaxic frame. A hole was then drilled in the parietal bone to access the VTA using stereotaxic coordinates. Glass micropipettes (6–18 M Ω) filled with 2.0% pontamine sky blue (BDH Chemicals, Poole, England) in 3M NaCl were used for recording. Signals were amplified using a Neuro Data IR283A (Cygnus Technology, Delaware Water Gap, PA), a Grass P15 (Astro-Med, West Warwick, RI) and a Neurolog System amplifiers (Digitimer, Hertfordshire, UK). Neuronal activity was filtered using cutting signal under of 300 Hz and over 3,000 Hz. The biopotentials were digitized using a Micro 1401 MK 2 Analogue-Digital Converter (Cambridge Electronic Design, Cambridge, UK) and stored on a computer using Spike 2 version 5.15 software (Cambridge Electronic Design, Cambridge, UK). Only spontaneously active VTA neurons were recorded and analyzed. VTA recordings were obtained from each animal using a sampling grid that extended 0.4–1.4 mm from midline, 7.0–9.0 mm from the brain surface and 4.9–5.7 mm caudal to bregma. Our recordings were performed between two physiologically defined boundaries to delineate the VTA. The dorsal limit was defined by the detection of neurons with short-duration action potentials and fast discharge rates (presumed GABA neurons), whereas the ventral boundary was defined by the detection of neurons with short-duration action potentials, and brief interspike intervals that are indicative of interpeduncular nucleus neurons. Neuronal activity was recorded for 3–5 min to establish baseline, and at least 5 min after drug infusion. Multiple cocaine injections in the same animal were made at least 45 min apart to permit metabolism of the drug prior to the next infusion. The recordings were performed at least 3 days after the implantation of the catheter surgery but not more than two weeks after the surgery.

Five to seven neurons were typically recorded per animal. The last recording site was marked by passing a 20 μ A cathodal current through the recording electrode for 10–15 min to deposit the pontamine sky blue dye. The brain was then removed and immersed in 10% formalin until it was sectioned (40 μ m) and mounted on slides to verify the recording site.

Experimental groups and drug dosage

Five groups of Long-Evans rats ($n = 6$ –12/group) were used in this study. Group 1 rats received i.v. infusions of 0.9% saline, the vehicle used for all drugs. In Group 2 and Group 3, each rat received cocaine-HCl (0.25 mg/kg), and one of these groups was pretreated with a mixture of D1- and D2-like DA receptor antagonists (SCH23390 and eticlopride, 1 mg/kg each s.c.) 20 min before the initiation of the recording session. In Groups 4 and Group 5, each rat received cocaine-MI (0.33 mg/kg), and one of these groups was pretreated with SCH23390 and eticlopride (at the same concentrations described above) 20 min prior to recording session. To maintain DA receptor blockade throughout the recording session, a half-dose of the initial antagonist mixture was administered at 3-hr intervals. This dosing schedule and concentration produces full dopamine blockade, as measured by antagonism of the striatal response to iontophoretic application of DA (Kiyatkin and Rebec, 1999). Each i.v. injection was given in a volume of 0.185 ml delivered over 10 s using a syringe pump. Cocaine-HCl and cocaine-MI solutions were slightly acidic (pH, 6.5 and 6.0, respectively). Saline vehicle pH was 7.0.

The dose of cocaine-HCl (0.25 mg/kg) was chosen because, while rats will still self-administer this concentration (Carroll & Lac, 1997; Wee et al., 2007), it is low enough to permit multiple injections in the same session. Our experimental animal received between 3 to 7 injections spaced with at least 45 min between injections. This interval is 4 times the half-life of cocaine (Booze et al., 1997). The dose of cocaine-MI was equimolar to the dose of cocaine. Cocaine-HCl and cocaine-MI were obtained from the National Institute on Drug Abuse Pharmacy Department. SCH23390 and eticlopride were purchased from Sigma Chemical Company (St. Louis, MO).

Population response—Impulse activity from individual neurons was summed every 5s, 1 min before to 5 min after drug infusion. Z-scores were computed using the mean and standard deviation of the discharge rate within the min of pre-drug activity. Individual neurons were grouped by drug treatment, and the mean and standard deviation of the z-scores were computed for each data bin. Mean z-scores were analyzed using repeated-measure ANOVA. The standard deviation of the z-scores was analyzed with a t-test for comparison of variance using the following formulas:

$$Sds = \sqrt{\frac{SD_1^2}{2n_1} + \frac{SD_2^2}{2n_2}} \quad \text{Standard Error}$$

$$t = \frac{SD_1 - SD_2}{Sds}$$

$$v = n_1 + n_2 - 2 \quad \text{Degrees of freedom}$$

Single neuron responses—Changes in spontaneous activity were analyzed by averaging ten 1s segments of impulse activity in a sliding window moving in 1s steps. Responses to cocaine analogs were defined as changes in firing rate of 20% above or below the spontaneous activity for at least 30s. The onset of response was the first bin within the 10s averaging window that was 20% above or below the mean spontaneous activity in the pre-drug period.

Because baseline impulse activity can vary during long-term recording, we validated the response criteria in two ways: (i) by determining how often discharge rates cross the threshold criteria of 20% above or below for at least 30s, and (ii) by calculating how often discharge rates cross the threshold criteria after i.v. saline injection (Table 1).

Statistics—Parametric statistics were used after verifying compliance for normal distribution and equal variance assumptions. Unpaired t-tests were used to compare between groups, and paired t-tests or two-way repeated measures ANOVAs were used to compare between groups and within group effects across time. Normality was assessed using the D'Agostino & Pearson omnibus normality test. Equality of variance was assessed with the Bartlett's test for equal variances. The sphericity of the matrix assumption for repeated-measures ANOVA was assessed with the Mauchley Sphericity Test; when the outcome of the test was significant the F values were corrected using the Greenhouse-Geisser approach (Glantz and Slinker, 2000). Fisher's LSD post-hoc test was used to establish mean differences after the ANOVA. A Kruskal-Wallis' test was used to compare between-group differences followed by Dunn's Multiple Comparison for non-parametric comparison. Fisher's exact test was used to compare proportions. A criterion of $p < 0.05$ was set as the level of significance.

Results

Central and peripheral contributions of cocaine to VTA neuron activity

We wanted to determine whether cocaine acting peripherally conveys an interoceptive signal to VTA neurons in cocaine-naïve animals. If so, cocaine should produce changes in the VTA neural activity immediately upon its administration. To test this hypothesis, VTA neurons were recorded and their discharge rates analyzed and plotted with respect to the initiation of either cocaine-HCl or cocaine-MI i.v. injections. Two population analyses were performed: changes in mean discharge rate and changes in the standard deviation of the discharge rate. The population mean discharge rate analysis tested for a dominant effect among all recorded neurons. However, since the mean population discharge rate would be zero if equally opposing effects were observed, we also analyzed the standard deviation to determine whether the pattern of VTA neuron activity changed, independent of direction of responses.

We determined whether the i.v. injection *per se* generates an interoceptive signal. We recorded 23 VTA neurons and examined their responses to i.v. 0.9% saline, the vehicle in which we dissolved our drugs. Figure 1A shows no changes in the mean discharge rate after i.v. saline. However, a small increase in the standard deviation of the discharge rate was observed beginning immediately after the i.v. infusion and was sustained for the 5 min of recording (Fig. 1C), suggesting that the i.v. administration procedure alone can contribute, albeit minimally, to changes in VTA cell activity.

Contrary to the weak effect evoked by saline, cocaine-MI and cocaine-HCl produced robust, rapid and longer-lasting changes in VTA neuron firing. Although population mean discharge rates after cocaine-MI or cocaine-HCl were not significantly different from baseline firing rates, there was an increase in discharge variability, as revealed by the increase in the

standard error of the means (Fig. 1B). The absence of a dominant change in VTA activity is caused by opposing effects of cocaine-MI or cocaine-HCl in individual neurons; neurons in both groups were either excited or inhibited by each drug (see examples in Fig. 2A). To determine when changes in VTA neuron activity occurred, we analyzed the timing of the changes in the standard deviations of the discharge rates. Both cocaine-MI and cocaine-HCl changed VTA neural activity within 5 s after the initiation of the drug infusion and this continued throughout the 5 min of post-injection recording (Fig. 1C).

To differentiate peripheral from central effects of cocaine, we compared the standard deviation of the discharge rate in neurons tested with cocaine-MI from those tested with cocaine-HCl. Near the time of the infusion changes in the standard deviation of the discharge rate were similar for both drugs. However, beginning at 75s after the start of the infusion differences between the drugs emerged. Whereas cocaine-MI reached its peak effect around the 70s mark and then slowly decreased, the effect of cocaine-HCl peaked at 90s and remained at this level before increasing further from 180s after the drug infusion (Fig. 1C). Thus, changes in VTA neuronal firing rates caused by cocaine-MI peaked during the first min post-infusion, whereas the effects of cocaine-HCl peaked 3 min after injection, and persisted longer. These extended actions of cocaine must be central since they were not observed with cocaine-MI.

Cocaine either decreases or increases VTA neuron discharge rate through actions in the peripheral nervous system

To further examine the patterns in discharge rates evoked by cocaine, we grouped neurons according to the direction of responses. When responsive cells are grouped together, cocaine-HCl evoked double the amount of responsive neurons than those observed with cocaine-MI (74% vs 35%, Table 1). Differences between cocaine-MI and cocaine-HCl were also observed in the onset of responses (Mantel-Haenszel log-rank test, $p < 0.001$; Fig. 2B). Similar proportions of responsive VTA neurons were observed within the first 45s after drug infusion with both cocaine analogs. However, beginning at 50s after the start of the injection the curves diverged, with cocaine-HCl continuing to evoke responses up to 3 min after injection, and cocaine-MI rarely continuing to alter firing rates from 1 min after drug infusion. Thus, peripherally triggered effects of cocaine occur within the first 50s while a later, centrally-generated effects, can be evoked up to 3 min after the drug injection. Regardless of cocaine formulation, no differences were found in mean latency to evoke increases or decreases in VTA neuron firing rates (Fig. 2C; Kruskal-Wallis' test, $p = 0.11$).

Neurons were subdivided into groups that were either inhibited or excited by cocaine-MI or cocaine-HCl. This categorization revealed that a significantly smaller proportion of neurons were inhibited by cocaine-MI (11/59, 19%) than by cocaine-HCl (30/74, 41%, Table 2). Conversely, no differences were found in the proportions of neurons excited by these drugs. In addition, 6 out of 74 neurons tested with cocaine-HCl showed combined responses comprised of either excitation followed by inhibition or inhibition followed by excitation. These neurons were excluded from further analysis. No biphasic responses were observed with cocaine-MI. We also observed no difference in proportion of neurons that responded to the drug on the first or subsequent injections. Most responses to cocaine-HCl were long-lasting exceeding the 5 minutes of recording and were not different after the first or subsequent injections. In neurons that were recorded long enough to see their activity return to baseline, this happened between 10 to 20 min after injection, within the time range reported previously by Einhorn et al (1988) at doses of 0.5 and 1 mg/kg.

VTA neurons that were inhibited by cocaine-HCl and cocaine-MI had similar response magnitudes starting 20 s after the initiation of the drug infusion. However, cocaine-MI inhibition abated after 3 min whereas cocaine-HCl inhibitions were maintained throughout

the 5 min of recording (Repeated Measure ANOVA within group $\times$ time ($F_{(3, 152)} = 9.71$; $p < 0.0001$, Greenhouse-Geisser corrected; $LSD < 0.05$ (Fig. 2D). Significant changes in the magnitude of excitation were first observed 50s after cocaine-HCl infusion and were maintained from 3 to 5 min. Cocaine-MI produced a sustained excitation from 2 to 5 min after the injection (Fig. 2E; $F_{(3, 152)} = 9.71$; $p < 0.0001$, Greenhouse-Geisser corrected; Fisher LSD post-hoc test, $p < 0.05$). No differences in response duration between groups were observed.

DA receptor activation contributes to both the peripheral and central effects of cocaine

We next tested whether changes in VTA neural activity evoked by cocaine-MI and cocaine-HCl were dependent on the activation of DA receptors. We pre-treated animals with a mixture of D1-like and D2-like DA receptor antagonists (SCH23390 and eticlopride, 1 mg/kg of each s.c.) to produce a full DA receptor blockade. No significant changes in mean discharge rate were found after the injection of cocaine-HCl or cocaine-MI. Nevertheless, a tendency toward excitation was observed for cells tested with cocaine-MI and cocaine HCl in pre-treated rats (Fig. 3A).

DA receptor blockade enhanced peripherally evoked cocaine neural VTA responses. When cocaine-MI was injected in the presence or absence of DA receptor blockade, the variability of the population discharge rate increased within the first min after the injection, but more robustly under DA receptor blockade (Fig. 3B). Interestingly, a large secondary increase in variability was observed 2 min after cocaine-MI infusion under these conditions. This was related to the peak effects observed in 6 of the 40 recorded neurons; 4 of them were excited while the other 2 were inhibited.

Contrary to the peripherally mediated actions of cocaine, DA receptor blockade attenuated cocaine's central effects. When DA receptors were blocked, the long-lasting changes in the discharge rate standard deviation previously seen with cocaine-HCl were substantially diminished (Fig. 3B). Moreover, the effect of cocaine-HCl on the standard deviation of the discharge rate during DA receptor blockade was similar to the long-lasting changes caused by cocaine-MI alone after a min after the injection had passed, suggesting that the DA antagonists greatly attenuated the central actions of cocaine. More evidence of this loss of centrally-mediated responses comes from the latencies of individually responsive neurons, which did not differ significantly between cocaine-MI and cocaine-HCl when animals were pre-treated with DA antagonists (Mantel-Haenszel log-rank test, $p > 0.05$; Fig. 4A and B).

Blockade of DA receptors attenuates cocaine-induced inhibition

Pre-treatment with DA receptor antagonists increased the proportion of cocaine-MI-evoked excitations, 18% to 43%, without affecting the proportion of inhibitions (Table 2). In contrast to the higher proportion of neurons responsive to cocaine-HCl versus cocaine-MI (55/74 and 21/59, respectively, Table 1), under DA receptor blockade the proportion of responsive neurons did not differ significantly between cocaine-HCl and cocaine-MI (29/40 and 22/44, respectively, Table 1).

Cocaine-MI and cocaine-HCl-evoked inhibitions were both weaker under DA receptor blockade. The onset of inhibition in animals pretreated with DA antagonists reached significance at a later time than that observed in untreated animals. In antagonist pretreated rats, the onset of inhibition caused by cocaine-MI was 30s, whereas the onset for the effects of cocaine-HCl occurred at 50s post-injection. This contrasts with the 20s latency of onset for both cocaine-MI and cocaine-HCl in untreated animals (Fig 4C; Repeated Measure ANOVA within group $\times$ time $F_{(3, 134)} = 8.41$; $p < 0.001$, Greenhouse-Geisser corrected). Moreover, cocaine-MI inhibitions were smaller in magnitude in animals pre-treated with DA

receptor antagonists without affecting its duration (Repeated Measure ANOVA between group $\times$ time $F(8, 134) = 2.97$; $p < 0.01$, Greenhouse-Geisser corrected). In neurons tested with cocaine-HCl under DA receptor blockade the inhibition abated 2 min after the injection, in contrast to the long-lasting inhibition in untreated rats (Fig 4C; Repeated Measure ANOVA within group $\times$ time $F(3, 134) = 8.41$; $p < 0.001$, Greenhouse-Geisser corrected).

The cocaine-induced excitation of VTA neurons reached significance with a shorter latency during DA receptor blockade (Two-way repeated measure ANOVA within treatment effect across time, $F(3, 159) = 15.96$; $p < 0.00001$, Greenhouse-Geisser corrected, Fisher LSD post-hoc test, $p < 0.05$). In pretreated rats, the excitation onset was 20 s in neurons tested with cocaine-MI and 40s for those neurons tested with cocaine-HCl. This contrasts with the tendency toward excitation with cocaine-MI and the 50s latency obtained for cocaine-HCl in untreated animals. In contrast to the latency, the duration of the excitation was not altered by pretreatment with DA receptor antagonists. Cocaine-MI elicited excitations from min 2 to the end of the min 5, in untreated animals, and elicited excitation 1 min after drug administration and a sustained activation from 3 to 5 min in the antagonist pretreated animals (Fig. 4D; $F(3, 152) = 9.71$; $p < 0.00001$, Greenhouse-Geisser corrected; Fisher LSD post-hoc test, $p < 0.05$). Similarly, cocaine-HCl during DA receptor blockade showed a sustained excitation from min 2 through the end of the 5 min recordings (Fig. 4D).

Peripheral actions of cocaine primarily affect putative DA neurons

The preceding data demonstrate that cocaine alters discharge rate of VTA neurons through its actions in the periphery. Therefore, we next sought to determine, whether these cocaine-responsive neurons demonstrate specific electrophysiological characteristics. Most VTA single-unit extracellular recordings are grouped based on distinguishing electrophysiological signatures: neurons displaying low discharge rates (< 10 Hz) and long-duration action potentials (> 2.5 ms) are typically classified as DA neurons, while cells with short-duration (< 2.5 ms) action potentials and high discharge rates (> 10 Hz) are classified as non-DA, presumably GABA neurons (Bunney et al., 1973; Wang, 1981; Grace and Bunney, 1983; Georges and Aston-Jones, 2002). We recorded from 240 VTA neurons whose action potential duration ranged from 1.08 to 5.30 ms with discharge rates ranging from 0.12 to 33.25 Hz. In general, cells with long-duration action potentials had lower discharge rates than those with short-duration action potentials ($r = -0.409$; $p < 0.0001$).

We found that the distribution of action potential durations in these neurons was biphasic, demonstrating a clear separation between groups at 2.2 ms (Fig. 5A). We used this 2.2 ms cutoff to group neurons into short- or long-duration action potential groups, and discharge rates ≥ 10 Hz were used to further divide these populations into low and high discharge rate groups. Neurons demonstrating long-duration action potentials (> 2.2 ms) and low discharge rates (< 10 Hz) were classified as putative DA neurons. Neurons with short-duration action potentials (≤ 2.2 ms) and high discharge rates (> 10 Hz) were classified as putative GABA neurons. However, these criteria did not completely describe the heterogeneity of VTA neurons, as 24% of the cells did not meet criteria for either group. These remaining cells had either short-duration action potentials with low discharge rates or long-duration action potentials with high discharge rates. These units were therefore classified as “unknown” phenotype neurons (Fig. 5C).

As indicated above, DA receptor antagonists did not change the overall discharge rate of VTA neurons. However, this treatment did shorten the action potential duration across all neurons (Fig. 5D). Although the duration of action potentials was shorter in animals pretreated with DA antagonists, the distribution of these durations still demonstrated a clear separation at 2.2 ms (Fig. 5D bottom middle and right panels), suggesting that the change in

action potential duration was not the result of a shift in the proportion of neurons in each of the classifications.

Independent of excitation or inhibition of activity by cocaine-MI, most of these responsive neurons (17/21, 81%) were classified as putative DA neurons, as defined by firing frequency and action potential duration criteria (top left quadrant, Fig 6). Of these, 9 were inhibited and 8 excited. No differences were observed in the duration of action potentials between neurons inhibited or excited by cocaine-MI. Similarly, in rats pretreated with DA receptor antagonists, most cocaine-MI responsive neurons (16/24, 66%) had long-duration action potentials with low discharge rates, characteristics associated with DA neurons (top left quadrant, Fig. 6 left panel). Of these, 5 were inhibited and 11 were excited by cocaine-MI. No differences were observed in the duration of action potentials between neurons inhibited or excited by cocaine-MI (Fig. 6).

Characteristics of cocaine-HCl responsive neurons were more varied than those observed with cocaine-MI, likely due to the peripheral, as well as central, effects of the drug. Most inhibited cells showed characteristics of putative DA neurons (26/30, 87%), and the remaining 4 neurons were classified either as putative GABA neurons or the unknown phenotype. Conversely, cocaine-HCl excited neurons had more diverse characteristics; 9 of 19 were classified as DA neurons, 5 were classified as GABA neurons and 5 were of unknown phenotype (Fig. 6). Comparison of action potential duration showed that cocaine-HCl-inhibited neurons possessed longer duration action potential than cocaine-HCl-excited neurons (One-way ANOVA, $F(2,54) = 4.749$, $p = 0.0128$, Newman-Keuls 0.01).

Under DA receptor blockade, most cocaine-HCl-inhibited neurons have characteristics of putative DA neurons (11/14, 79%). Cocaine-HCl-excited neurons had diverse characteristics; 7 of 17 were classified as putative DA neurons, 8 were of unknown phenotype with short-duration action potentials and low discharge rates, and the remaining 2 were of unknown phenotype with long-duration action potentials and high discharge rates (Fig. 6). Similar to the untreated condition, during DA receptor blockade, the action potential duration of cocaine-HCl-inhibited neurons were longer in duration than that in cocaine-HCl-excited neurons (Unpaired t-test, $t=2.383$, $p = 0.024$).

Discussion

The present study demonstrates that cocaine acts in the periphery producing a rapid interoceptive signal capable of altering VTA neuron activity prior entering the brain and interacting directly with VTA neurons. These data also support the existence of a synaptic pathway through which cocaine's actions in the periphery are communicated to VTA neurons. Both excitatory and inhibitory inputs to VTA neurons are altered by cocaine's actions in the periphery. DA receptors modulate cocaine's peripheral-evoked interoceptive signal decreasing excitatory inputs while enhancing inhibitory inputs.

Methodological Considerations

Our current understanding of the cellular makeup of VTA is rapidly expanding. In addition to better-described DA and GABA neurons, the VTA also contains glutamate neurons (Steffensen *et al.*, 1998; Yamaguchi *et al.*, 2007; Yamaguchi *et al.*, 2011). Both GABA and glutamate VTA neurons synapse onto DA neurons; and although DA neurons do not make many local synapses, somatodendritic release of DA may locally regulate GABA and glutamate VTA neurons (Omelchenko and Sesack, 2009; Dobi *et al.*, 2010). This heterogeneity of cellular phenotypes highlights the importance of studying all VTA neurons independently of their presumed neurochemical nature. Moreover, recent work revealed that indirect electrophysiological identification of DA neurons may not be accurate since some

electrophysiologically identified DA neurons were found to be non-DA (Cameron *et al.*, 1997; Ungless *et al.*, 2004; Margolis *et al.*, 2006; Luo *et al.*, 2008)

Because of the heterogeneity among VTA neurons, we recorded every neuron encountered within the VTA and tested its response to cocaine. This differed from previous experimental designs in which cells were pre-selected based on a combination of electrophysiological criteria (Einhorn *et al.*, 1988; Marinelli and White, 2000; Marinelli *et al.*, 2003; Zhou *et al.*, 2005; Steffensen *et al.*, 2008). Using this approach, we were able to detect the population response of cocaine of the VTA as a structure. After this, we subdivided recorded neurons into groups according to whether they were inhibited or excited by cocaine. Finally, we described the electrophysiological characteristics of cocaine-responsive neurons.

Although any anesthetic may affect the activity and responsiveness of neurons (Windels and Kiyatkin, 2006), we selected to record under anesthesia based on several reasons: (1) it is a long-established preparation in which VTA responses to i.v. cocaine had been studied (Einhorn *et al.*, 1988; Marinelli and White, 2000; Marinelli *et al.*, 2003; Zhou *et al.*, 2005; Steffensen *et al.*, 2008); (2) it allows for good quality single-cell recording during injection of cocaine; (3) it served to identify the mechanism of drug actions that under awake condition can be hard to separate from behavioral and motor effects of the drug. Urethane was used because it has been proved to be a good preparation to study sensory effects on midbrain DA neurons (Chiodo *et al.*, 1980).

VTA population response to i.v. cocaine

We found that i.v. cocaine either excited or inhibited individual VTA neurons. These responses were opposite in direction and often similar in magnitude resulting in no significant changes in mean discharge rate. Because of the underlying dichotomy of responses, we analyzed the standard deviation of the discharge rate to characterize the effects of cocaine regardless of the direction of the changes in activity of each individual neuron. Using this approach, we found that VTA neuronal changes started rapidly within 5s from the start of 10s injection. The similarity in the time-course and magnitude of change induced by cocaine-HCl and cocaine-MI tight to the time of injection indicates that cocaine-induced VTA changes originate from cocaine's action in the periphery. This rapid peripheral action of cocaine began to decline ~90s after the injection and was followed by a robust and long-lasting central action that continued beyond the 5 min recording interval used in this study. In contrast to our approach, previous studies that have pre-selected for putative DA or GABA neurons had reported individual responses and mean responses within these categories (Einhorn *et al.*, 1988; Marinelli and White, 2000; Marinelli *et al.*, 2003; Zhou *et al.*, 2005; Steffensen *et al.*, 2008). These authors reported mainly an inhibitory effect on putative DA and excitatory effect on putative GABA neurons; however, when all neurons are examined, as in our case, no dominant VTA response was observed. Our findings highlight that VTA responses to cocaine are fast, heterogeneous, start in the periphery, and that both excitations and inhibitions have similar weights on the VTA population response.

Cocaine-induced VTA neuron inhibition

Cocaine-induced inhibition of VTA neurons was initiated through cocaine's peripheral actions but was enhanced by its central actions. Both cocaine-MI- and cocaine-HCl-evoked inhibitions were temporally correlated, started within 20 s of the initiation of injection and had similar magnitudes. However, the inhibition produced by cocaine-MI abated by 3 min post-injection, whereas the cocaine-HCl responses persisted throughout the duration of the 5-min recording. The proportion of neurons inhibited by cocaine-HCl was also higher than that observed with cocaine-MI. This suggests that there are likely two independent groups of inhibited neurons affected by cocaine, one that receives an inhibitory input evoked by

cocaine's peripheral actions, and a second, whose inhibition is generated by a central mechanism. Evidence in favor of this dichotomy is that the onset latency of the inhibition was delayed in some neurons tested with cocaine-HCl but not on those tested with cocaine-MI.

Blockade of both D1- and D2-like DA receptors with antagonists also revealed a differential involvement of these receptors in the peripheral and central effects of cocaine. Following DA receptor blockade, the magnitude of cocaine-MI induced inhibitions was smaller, and the inhibition caused by cocaine-HCl did not persist as long as that observed in the absence of the antagonists. Interestingly, most of the cocaine-inhibited neurons were putative DA neurons as they demonstrated long-duration action potentials and low discharge rate (Bunney *et al.*, 1973; Wang, 1981; Grace and Bunney, 1983; Georges and Aston-Jones, 2002). However, because we did not observe changes in the latency of onset of inhibition between cocaine-MI and cocaine-HCl, either in the presence or absence of DA antagonists, this suggests that the critical site for DA receptor blockade is in the CNS. Thus, the longer duration of inhibition of VTA neuron activity with cocaine-HCl in the absence of DA receptor blockade might reflect a central drug action that increases local levels of DA in the VTA and results in feedback inhibition through D2 autoreceptors on VTA DA neurons (Einhorn *et al.*, 1988; Lacey *et al.*, 1990; Cameron *et al.*, 1997).

The source of inhibitory inputs to the VTA that arise from the periphery may be derived from interoceptors relaying visceral sensory information originating from internal organs through the parasympathetic and sympathetic afferent systems that sends information to the nucleus of the solitary tract and the parabrachial nucleus (Saper, 2002), each of which sends afferents to the VTA (Geerling and Loewy, 2006; Geisler *et al.*, 2007; Mejías-Aponte *et al.*, 2009). Electrical stimulation of either of these brain stem areas can inhibit VTA neuron activity (Kirouac and Ciriello, 1997; Coizet *et al.*, 2010). Another source of inhibition of VTA neurons is the nucleus accumbens, which is activated by input from the nucleus of the solitary tract and the ventrolateral medulla (Kirouac and Ciriello, 1997), and sends inhibitory GABAergic projections to the VTA (Xia *et al.*, 2011). Interestingly, the nucleus accumbens neurons are activated by cocaine-MI (Kiyatkin and Brown, 2007), further suggesting that the nucleus accumbens might be involved in transmitting the cocaine signal from the periphery to the VTA.

Cocaine-induced VTA excitations

Cocaine-MI-induced excitations were similar to those observed with cocaine-HCl. Furthermore, the duration of this excitation was independent of DA receptor activation, and it lasted throughout the 5 min recording period. The proportions of cocaine-MI and cocaine-HCl-excited neurons were also similar, although the characteristics of the responding neurons were different. Thus, most neurons excited by cocaine-MI were putative DA neurons (80%), whereas those excited by cocaine-HCl were more diverse in their electrophysiological characteristics, with only 48% classified as putative DA neurons. Of the 52% putative non-DA neurons excited by cocaine, 26% were putative GABA neurons, with electrophysiological characteristics similar to those described previously by Steffensen and colleagues (Steffensen *et al.*, 1998; Steffensen *et al.*, 2008). Similar to inhibitions, some excitations induced by cocaine-HCl had delayed onset latencies, indicating that some could be generated centrally. The fact that the electrophysiological characteristics of the excited VTA neurons were different between cocaine-MI and cocaine-HCl suggests that the peripheral actions of cocaine preferentially target putative DA neurons, and when cocaine acts centrally a subgroup of non-DA neurons is also affected.

The excitations of VTA neurons induced by the two cocaine analogs had shorter latencies when DA receptors were blocked. However, the ubiquitous presence of DA receptors in the

peripheral and central nervous systems makes it difficult to differentiate whether this effect resulted from a central or peripheral receptor blockade. In the sympathetic terminals, norepinephrine release can be increased by blocking D2 receptors (Ilhan and Long, 1975); thus, when cocaine blocks the norepinephrine transporter, sympathetic actions of norepinephrine are further increased and might occur at earlier times. Centrally, DA receptor blockade might enhance glutamate release from afferents in the VTA such as those derived from the parabrachial or the nucleus of the solitary tract (Geisler and Zahm, 2005; Geerling and Loewy, 2006; Geisler et al., 2007; Mejías-Aponte et al., 2009), where DA exerts an inhibitory role over incoming inputs to these nuclei (Chen *et al.*, 1999; Kline *et al.*, 2002). Moreover, inhibition of presynaptic inhibitory D2 control of glutamate release in the VTA might also play a prominent role in the enhancement of excitatory inputs under DA receptor blockade (Koga and Momiyama, 2000).

The increase in discharge rate evoked by cocaine can also be explained by a decrease in tonic inhibitory inputs to VTA neurons. The mesopontine rostral tegmental nucleus, a major GABAergic input to VTA neurons (Jhou et al., 2009), is tonically inhibited by acute i.v. cocaine (Lecca et al., 2011). This structure receives afferents from the nucleus of the solitary tract and the parabrachial nucleus, making it a likely candidate through which peripheral information may be forwarded to VTA neurons (Jhou et al., 2009; Kaufling et al., 2009).

Peripheral effects of i.v. cocaine are mediated by two discriminable processes and are dependent on the state of the animal

One effect of our i.v. cocaine injections was completely independent of the pharmacological actions of the drug. Smirnov and Kiyatkin (2010) have shown that i.v. saline injections cause changes in cortical EEG immediately after the injection and that temporally correlates with the changes caused by i.v. injections of cocaine-HCl or cocaine-MI, but abate within a min while the effects of both cocaine analogs remained for more than 5 min. We found a similar effect on VTA neural activity; i.v. saline injection increases the standard deviation of the mean impulse activity of VTA neurons with similar onset latency to those observed with cocaine-MI and cocaine-HCl but the effect was much weaker in magnitude. It is important to highlight that saline does not produce distinguishable long-lasting responses at the single-neuron level (see Table 1) and only when all neurons are pulled together we observed changes in variability of the VTA neural activity. Therefore, it is clear that the i.v. infusion of cocaine has at least two effects on VTA neuron activity; one initiated by some aspect of the i.v. injection unrelated to cocaine, and the other mediated by the pharmacological actions of cocaine.

The effects of i.v. cocaine are also dependent upon the state of the animal. Under urethane anesthesia, the time course of cocaine-HCl induced cortical desynchronization is delayed and saline does not desynchronize cortical activity (Kiyatkin and Smirnov, 2010). In the present study, conducted in urethane anesthetized rats, the changes in VTA neuronal activity began 5 s after initiation of the injection and significant inhibition and excitation occurred at 20s and 50s post-injection, respectively. This contrasts with the more immediate excitations of VTA neurons observed in awake rats [(4–6 s, after the initiation of the injection (Brown and Kiyatkin, 2008)]. However, in the latter study, the effect of i.v. saline injection was not investigated; making unclear what parts of the responses are due to cocaine pharmacological actions or to the sensation of the i.v. drug delivery. Nevertheless, it is clear that i.v. administration of cocaine initially produces a fast sensory-evoked component tied to the i.v. drug delivery that depends on the arousal state of the animal. This peripherally triggered action is followed by a slightly delayed pharmacological action of cocaine that also originates in the periphery. Both, the fast sensory-evoked component and the peripheral pharmacological actions of cocaine alter VTA neuron activity and are followed by a prolonged direct effect of the drug in the brain.

The source of cocaine's peripheral signals to VTA neurons

The source of cocaine's peripheral actions that produces changes in afferent inputs to the VTA is unknown. However, VTA receives inputs from brain regions that process the incoming sensory information from the peripheral nervous system including the nucleus of the solitary tract, the ventrolateral medulla and the parabrachial nucleus (Geisler and Zahm, 2005; Geerling and Loewy, 2006; Geisler et al., 2007; Mejías-Aponte et al., 2009). Thus, is it likely that a change such as a raise in blood pressure might be forwarded from the periphery to the VTA. Evidence of an interaction between the cardiovascular system and VTA neural activity has been investigated by Kirouac and Ciriello (1997). Similar to our findings with cocaine, a raise in blood pressure can either excite or inhibit VTA neurons. Moreover, after cutting the aortic depressor and vagus nerves, the VTA responses to a raise in blood pressure were no longer observed; thus, indicating that sensory information from the periphery is needed to evoke these responses.

The cardiovascular system might be one of putative peripheral pathways through which cocaine influences the VTA neuronal activity. It is known that cocaine produces a raise in blood pressure. Concomitant to the blood pressure spike there is a decrease in heart rate (Poon and van den Buuse, 1998) that suggests baroreceptor activation. Although a vast literature on the cardiovascular effects of cocaine does exist, there is disagreement whether the cardiovascular response of cocaine is initiated in the central nervous system (Knuepfer and Branch, 1992; Tella et al., 1993) or in the periphery (Dickerson et al., 1999). For example, it can be suggested that cocaine produces the raise in blood pressure by increasing sympathetic output through the blockade of the norepinephrine transporter. This view can be strengthened by the fact that the raise in blood pressure induced by cocaine is blocked by alpha-1 adrenoceptor antagonist (Poon and van den Buuse, 1998). However, under this scenario, cocaine should have a fast access to sympathetic afferents that are located in the adventitia of blood vessels. Diffusion from the inside of the vessel to the adventitia takes approximately 60s as measured by physiological responses to cocaine on aortic baroreceptor activity (Andresen et al., 1990), which is more than time the onset of cocaine-induced raise in blood pressure, which is about 10–15s (Pitts et al., 1987; Poon and van den Buuse, 1998). Other evidence against this view is that cocaine inhibits but not activates baroreceptors by an adrenoceptor-independent mechanism (Andresen et al., 1990). A more direct evidence that the norepinephrine transporter is not a primary target of cocaine's peripheral signal to VTA neurons is that i.v administration of reboxetine (noradrenergic reuptake blocker) does not produce changes in discharge rate of VTA neurons (Linner et al., 2001).

In addition to its monoamine transporter blocker properties, cocaine can also blocked voltage gated Na⁺ and K⁺ channels, and Ca²⁺-activated K⁺ channels (Reith et al., 1986; Crumb and Clarkson, 1990; Ritz et al., 1990; Zhang et al., 2002; Premkumar, 2005; Kobayashi et al., 2007). Procaine, like cocaine-HCl and cocaine-MI, produces cortical synchronization with a fast response onset (~4s), indicating that non-monoamine properties of cocaine might be important for its peripheral actions (Smirnov and Kiyatkin, 2010). Cocaine can also act directly in the heart changing its excitability. In the atrium-isolated preparation cocaine decreases the rate of contractility in a monoamine-independent manner (Trendelenburg, 1968). Similarly, procaine decreases heart rate when delivered directly at the sinoatrial node through the sinus node artery (Satoh, 1981). The negative chronotropic effect of procaine on sinoatrial cells is a combination of blockade of sodium and potassium conductances (Satoh and Hashimoto, 1986). Thus, it is possible that the bolus infusion of cocaine might change heart conductivity leading to activation of compensatory mechanisms, such as changes in baroreceptor activity, and producing a peripheral signal that it is able to alter the activity of VTA neurons. When given i.v., procaine is capable to activate putative VTA DA neurons (Einhorn et al., 1988), thus supporting the view that

excitatory inputs to VTA neurons can be relayed through a non-monoamine mediated mechanism.

Conclusion

A recent microdialysis study showed that cocaine's actions in the peripheral nervous system give rise to cues that could be associated with the central effects of cocaine, thus gaining the ability to trigger DA and glutamate release in the VTA and reinstate cocaine-seeking habits (Wise *et al.*, 2008). Our present findings demonstrate that cocaine's actions in the peripheral nervous system can affect VTA neuron activity in drug-naïve rats, and we propose that following repeated drug use these effects may serve as interoceptive cues that might be relevant for the development of conditioned associations between the drug and any other environmental stimuli.

Highlights

- Cocaine either excites or inhibits VTA neurons.
- A peripheral acting cocaine analog excites or inhibits VTA neurons.
- Dopamine receptor blockade attenuates cocaine-induced inhibitions but enhances excitations.
- Cocaine modulates VTA neural activity before it enters the brain.

Abbreviations

BBB	blood-brain barrier
cocaine-HCl	cocaine-hydrochloride
cocaine-MI	cocaine-methiodide
DA	dopamine
DAT	dopamine transporter
i.v.	intravenous
VTA	ventral tegmental area

Acknowledgments

This work was supported by the National Institute on Drug Abuse, Intramural Research Program. We thank Cameron Good, Carl Lupica and Roy Wise for their insight and comments on the manuscript and for their helpful editorial assistance.

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Figure 1. Both cocaine-MI and cocaine-HCl produces immediate and long-lasting changes in VTA neuronal activity

(A) Population mean discharge rate of VTA neurons in rats intravenously infused with saline. (B) Population mean discharge rate of VTA neurons in rats intravenously infused with cocaine-MI and cocaine-HCl. No significant changes are observed in mean discharge rate; however, as the time after injection progresses, there is a heightened variability as shown in the size of the SEM. (C) Both cocaine-MI and cocaine-HCl have similar increase in standard deviation immediately after the initiation of the i.v. injection within 1 minute post-injection. Plot represents changes in discharge rate standard deviation of VTA neurons during 5 minutes post-injection recording at 5 s time resolution. Filled symbols are time

points significantly different from pre-drug impulse activity. Asterisks, *, represent times where cocaine-HCl was significantly different from cocaine-MI. Gray bars indicate the duration of the i.v. drug infusion.

Figure 2. Cocaine either excites or inhibits VTA neurons

(A) Representative examples of VTA neurons response to cocaine-MI and cocaine-HCl. Notice the changes in pace of the impulse activity before (from -15 to 0 s) and after the injection (from 10 to 45 s). (B) Cumulative distributions of latency onset of cocaine-MI and cocaine-HCl single-neuron VTA responses. Latency of the response onset of cocaine-MI responsive neurons is similar to those observed in cocaine-HCl close to the time of injection (within the 45 s). Most cocaine-MI responses happened before 80 s while cocaine-HCl continues to generate responses well into the third minute. (C) Latency onset of cocaine-MI and cocaine-HCl evoke excitations and inhibitions. Onset of single neuron responses is independent of response type. (D) Cocaine-MI evoked inhibitions have similar onset as

those evoked by cocaine-HCl, but has shorter durations. Top panel is a representative example of a cell inhibited by cocaine-MI. Bottom panel is the percent change that occurred with the first minute (left side of the figure) and during the 5 minutes of recording (right side of the figure). **(E)** Cocaine-MI and cocaine-HCl evoked excitations have similar onset and duration. Top panel is a representative example of a cell excited by cocaine-MI. Bottom panel is the percent change that occurred with the first minute (left side of the figure) and during the 5 minutes of recording (right side of the figure). Filled symbols are time points significantly different from within group pre-drug impulse activity. Gray bars indicate the duration of the i.v. drug infusion.

Figure 3. DA receptor blockade enhances cocaine peripheral contributions to VTA inputs but attenuates cocaine central actions

(A) Under DA receptor blockade (1 mg/kg of each SCH23390 and eticlopride, s.c.), Cocaine-MI exhibited a greater tendency toward excitation while cocaine-HCl's early tendency toward inhibition reverted toward excitation. Plots represent the population mean discharge rate in rats that received cocaine-MI (left panel) or cocaine-HCl (right panel) either in the presence or absence of DA receptor blockade. (B) Cocaine-MI increases discharge rate standard deviation immediately after drug injection (left panel). Cocaine-HCl long-lasting population response was attenuated under DA receptor blockade (right panel). Plots represent changes in discharge rate standard deviation of VTA neurons during 5 minutes post-injection recording at 5 s time resolution. Filled symbols are time point significantly different from pre-drug impulse activity. Asterisks, *, represent times where cocaine-MI was different from cocaine-MI under DA receptor blockade. Crosses, †, represent times where cocaine-HCl was different from cocaine-HCl under DA receptor blockade. Gray bars indicate the duration of the i.v. drug infusion.

Figure 4. DA receptor blockade attenuates cocaine evoked inhibitions

(A) Cumulative distributions of latency onset of cocaine-MI and cocaine-HCl single-neuron VTA responses in animals pretreated with DA antagonists. Most responses started within the first minute after the initiation of drug injection. (B) Latency onset of cocaine-MI and cocaine-HCl evoke excitations and inhibitions. Onset of single neuron responses is independent of response type. (C) Top panel shows an example of a cell inhibited by cocaine-MI under DA receptor blockade. Duration of cocaine-HCl-induced inhibitions was attenuated (middle panel). Magnitude of cocaine-MI-induced inhibition was smaller under DA receptor blockade (bottom panel). (D) Top panel shows an example of a cell excited by cocaine-MI under DA receptor blockade. DA receptor blockade did not affect cocaine-HCl-

induced excitation (middle panel). Under DA receptor blockade cocaine-MI-induced excitation had a tendency to occur earlier (bottom panel). Filled symbols are time points significantly different from within group pre-drug impulse activity. Asterisks, *, represents different from cocaine-MI. Gray bars indicate the duration of the i.v. drug infusion.

Figure 5. Categorization of VTA neurons according to action potential duration and discharge rate

(A) The distribution of action potential durations is continuous and has a valley around 2.2 ms. Left: A sample waveform measure from the beginning to the end of the action potential. Right: Histogram of action potential durations. (B) Long-duration action potential and low impulse activity were the more prevalent characteristics among the recorded neurons. Cells were divided based upon action potential durations and discharge rate (imp/s). Notice that 24% of the recorded neurons did not align with the “classic” description of a putative DA or GABA neuron. (C) Scatter plot of discharge rate vs action potential duration. Dotted lines are placed at 10 imp/s on the x-axis and 2.2 ms on the y-axis. The scatter plot is divided into

quadrants as follows: the gray shaded top left quadrant includes cells with long-duration action potentials and low discharge rate. This group of cells is considered putative DA neurons. Bottom left quadrant includes neurons with short-duration action potentials and low discharge rate. Top right quadrant includes cells with long-duration action potentials and high discharge rate. Bottom right quadrant includes cells with short- duration action potentials and high discharge rate. These are considered putative-GABA neurons. **(D)** DA receptor blockade did not change the mean discharge rates, but reduced action potential durations. Top left panel, discharge rates of neurons tested with cocaine-HCl and cocaine-MI in the presence or absence of DA antagonists. Top middle and right panel are cumulative distribution of action potential duration of cocaine-HCl and cocaine-MI tested neurons in the presence or absence of DA antagonists. Notice that DA receptor antagonists shift the distributions toward the left. Bottom left panel, comparison of duration of action potentials in the presence or absence of DA antagonists. Bottom middle panel shows a histogram of action potential duration of neurons recorded without DA receptor blockade. Bottom right panel shows a histogram of action potential duration of neurons recorded under DA receptor blockade. Both distribution have a valley at 2.2 ms.

Figure 6. Cocaine-MI responsive neurons have characteristic of putative DA neurons

The two top panels show the characteristics of cocaine-MI responsive neurons in the presence or absence of DA antagonists. Most cocaine-MI responsive neurons have long-duration action potential and low discharge rates; characteristics associated with putative DA neurons. Action potential duration did not differ between cocaine-MI excited and inhibited neurons. The two bottom panels show characteristics of cocaine-HCl responsive neurons in the presence or absence of DA antagonists. Most cocaine-HCl inhibited neurons have characteristics of putative DA neurons while cocaine-HCl excited neurons do not possess distinctive action potentials and discharge rates characteristics. Inhibited neurons have longer action potential duration than excited neurons.

Table 1

Proportion of neurons showing changes in discharge rate exceeding response threshold

Experimental Groups	Proportion of neurons that crossed threshold criteria	
	Baseline	Drug
Saline	4/23 (17%)	3/23 (13%)
Cocaine-HCl	10/74 (14%)	55/74 (74%) ^{a, b}
Cocaine-MI	6/59 (10%)	21/59 (35%) ^{a, b}
<i>DA receptor blockade</i>		
Cocaine-HCl	13/44 (31%)	29/44 (69%) ^{a, b}
Cocaine-MI	8/40 (20%)	22/40 (55%) ^{a, b}

^a, within treatment difference between baseline and drug proportions;^b, different from saline drug period proportions

Table 2

Proportion of excited and inhibited neurons after i.v. administration of cocaine-MI and cocaine-HCl

Response type	Cocaine-HCl n = 74	Cocaine-MI n = 59	Cocaine-HCl DA receptor blockade n = 44	Cocaine-MI DA receptor blockade n = 40
Inhibition	30 (41%)	11 (19%) ^a	14 (32%)	7 (18%) ^{a, b}
Excitation	19 (26%)	10 (17%) ^b	17 (39%)	17 (43%)

^a, different from cocaine-HCl inhibition;^b, different from cocaine-MI DA receptor blockade excitation