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Differential Effects of Cocaine on Dopamine Neuron Firing in Awake and Anesthetized Rats

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Cocaine (benzoylmethylecgonine), a natural alkaloid, is a powerful psychostimulant and a highly addictive drug. Unfortunately, the relationships between its behavioral and electrophysiological effects are not clear. We investigated the effects of cocaine on the firing of midbrain dopaminergic (DA) neurons, both in anesthetized and awake rats, using pre-implanted multielectrode arrays and a recently developed telemetric recording system. In anesthetized animals, cocaine (10 mg/kg, intraperitoneally) produced a general decrease of the firing rate and bursting of DA neurons, sometimes preceded by a transient increase in both parameters, as previously reported by others. In awake rats, however, injection of cocaine led to a very different pattern of changes in firing. A decrease in firing rate and bursting was observed in only 14% of DA neurons. Most of the other DA neurons underwent increases in firing rate and bursting: these changes were correlated with locomotor activity in 52% of the neurons, but were uncorrelated in 29% of them. Drug concentration measurements indicated that the observed differences between the two conditions did not have a pharmacokinetic origin. Taken together, our results demonstrate that cocaine injection differentially affects the electrical activity of DA neurons in awake and anesthetized states. The observed increases in neuronal activity may in part reflect the cocaine-induced synaptic potentiation found *ex vivo* in these neurons. Our observations also show that electrophysiological recordings in awake animals can uncover drug effects, which are masked by general anesthesia.

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INTRODUCTION

Cocaine (benzoylmethylecgonine) is a naturally occurring alkaloid extracted from plants belonging to the *Erythroxylum* species. It is known to produce a number of pharmacological effects, including psychomotor stimulation, hypertension, tachycardia, anorexia, and pupillary dilation. Central effects of cocaine are attributed to its ability to inhibit reuptake of dopamine, serotonin, and norepinephrine (Reith *et al*, 1986, 1997b; Richelson and Pfenning, 1984; Ritz *et al*, 1990; Ross and Renyi, 1967). It is believed that blockade of the uptake, by increasing the synaptic concentrations of these neurotransmitters (Bradberry and Roth, 1989; Li *et al*, 1996; Reith *et al*, 1997a), leads to an increase in vigilance and sensory awareness, activation of defense mechanisms, cognitive distortion, euphoria, and a reduced need for sleep (Clark *et al*, 1985b; Freye and Levy, 2009).

Owing to its potent rewarding effect, cocaine has a high abuse liability. Its consumption in modern society has become increasingly common, as shown by population statistics (Clark *et al*, 1985b; Freye and Levy, 2009). Consequences of chronic use include a massive addiction, an increased risk of psychiatric illnesses, and deleterious consequences on general health (eg, an increased risk of myocardial infarction) (Lange and Hillis, 2001).

The addictive properties of cocaine are thought to be mediated mainly by the dopaminergic (DA) mesocorticolimbic system—the pathways projecting from the ventral tegmental area (VTA) to the medial prefrontal cortex, nucleus accumbens (NAc), amygdala, and hippocampus (Chiodo *et al*, 1984; McCutcheon *et al*, 2009; Peoples *et al*, 1998; Wise and Bozarth, 1984). Using *in vivo* voltammetry, it was shown that systemic cocaine injection in awake and behaving rats produces a significant increase of DA

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concentration in NAc synapses, correlating to the cocaine-induced psychostimulant behaviors (Broderick *et al*, 1993). On the other hand, application of cocaine in electrophysiological experiments leads to a decrease in the firing of DA neurons *in vivo* (Einhorn *et al*, 1988; Hinerth *et al*, 2000; Mercuri *et al*, 1992) and a hyperpolarization of DA neurons *in vitro* (Brodie and Dunwiddie, 1990; Einhorn *et al*, 1988; Lacey *et al*, 1990; Mercuri *et al*, 1991, 1992). These effects are explained by the fact that dopamine, released from the axons and dendrites, enhances the activity of an NAc-VTA GABAergic negative feedback and activates somatodendritic autoreceptors on the DA neurons (Einhorn *et al*, 1988; Kalivas, 1993; Wang, 1981; White and Wang, 1984a, b, 1986). However, *in vivo* electrophysiological experiments mentioned above were conducted under general anesthesia, which is known to change the responses of central neurons to various compounds (Kelland *et al*, 1990, 1989; Nicoll and Madison, 1982; Windels and Kiyatkin, 2006). In particular, it was shown that anesthesia affects cocaine metabolism (Benuck *et al*, 1989; Pan *et al*, 1995), alters the reactivity of DA neurons to glutamate, GABA, DA agonists, and DA antagonists (Bunney *et al*, 1973a; Clark *et al*, 1985a, b; Gessa *et al*, 1985; Kelland *et al*, 1990, 1989; Melis *et al*, 1998; Mereu *et al*, 1984, 1987; Windels and Kiyatkin, 2006), and interferes with DA turnover (Westerink *et al*, 1977). For example, nicotine and ethanol have a stimulating effect on the VTA neurons in awake rats, but fail to activate them in anesthetized animals (Gessa *et al*, 1985; Mereu *et al*, 1987). A number of combined electrophysiological and behavioral studies on cocaine have been performed in awake rats, but have mostly focused on NAc neurons (Carelli, 2002; Carelli and Deadwyler, 1994; Peoples *et al*, 1998; Peoples and West, 1996; Stuber *et al*, 2005).

On the other hand, *in vitro* investigations of the cocaine effect on VTA neurons were performed in slices, where some important regulatory pathways are severed. These caveats make it difficult to compare cocaine-induced electrophysiological effects in the midbrain and its behavioral actions. Thus, the question of how cocaine administration changes the activity of DA neurons in awake, behaving animals remains open.

To address this issue, we measured the effects of this drug on the firing rate and pattern of midbrain DA neurons in both awake (and freely moving) and anesthetized rats, taking advantage of the recent development of telemetric techniques. In addition, we measured plasma and brain concentrations of cocaine and its main metabolite, benzoylecgonine (BZE) (Freye and Levy, 2009), in order to evaluate the possibility of differential pharmacokinetics of cocaine in the two conditions and to measure the actual brain concentrations after the injection of a behaviorally relevant dose of the drug.

MATERIALS AND METHODS

Subjects

Adult male Wistar rats, weighing 290–310 g at the time of surgery, were housed individually and maintained on a 12-h light : 12-h dark cycle. Water and food were available *ad lib*. All animal care and handling was conducted in accordance with the guidelines stated in the Handbook for the Use of Animals in Neuroscience Research (Society for Neuroscience, 1991). All procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EE) and were approved by the Ethics Committee for Animal Use of the University of Liege (protocol 86).

Surgical and Histological Procedures

Rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneally (i.p.)), and placed in a stereotaxic apparatus (Model 902, Kopf). Soft tissues of the skull were anesthetized using a subcutaneous injection of 0.5% lidocaine hydrochloride. Additional supplemental doses of chloral hydrate and/or lidocaine were injected when necessary. Body temperature was maintained at 36–37°C by means of a heating pad. The skin was retracted, and the exposed bone cleaned with saline and a 3% hydrogen peroxide solution. Four to five small holes were made in the skull around the area of entry using a 1.10 mm diameter dental burr (Microtorque II control box, Tech 2000 Handpiece, Ram Products, Encino, CA), and small stainless-steel screws ($1.20 \times 3.15\,mm^2$) were screwed to subsequently anchor the microelectrode array (MEA) to the skull using dental restorative material.

The area of entry was defined according to stereotaxic coordinates (Ford and Williams, 2008). A small part of the skull between the lambda and the bregma was removed above the implantation point using a 0.4 mm diameter dental burr. An MEA (8 recording and one reference electrode; Supplementary Figure S1) was mounted on the micromanipulator and the tips of the electrodes were lowered into the VTA through the opening (5.8–6.8 mm posterior to the bregma, 0.6–0.9 mm lateral to the midline, and 7.5–8.5 mm under the cortical surface; Supplementary Figures S1 and S2A). The MEA was fixed to the rat's skull and the supporting screws using Z100 dental restorative and Adper Scotchbond multi-purpose adhesive (3M ESPE Dental Products, St. Paul, MN). A scheme representing the geometry of the electrodes in the MEA is shown in Supplementary Figure S1B.

At the end of the recordings, the rats were deeply anesthetized and perfused intracardially with saline, followed by a 4% formaldehyde saline solution. The brains were removed and stored at 4°C in a 4% formaldehyde/sucrose solution. Frozen sections (80 μm) were cut, mounted, and stained with cresyl violet for histological examination (Gold *et al*, 2006). In all experiments reported here, this examination confirmed that the recording electrodes were positioned within the dorsocaudal section of the VTA (Supplementary Figure S2A and B). According to tracing experiments, the caudal VTA contains a higher proportion of DA than GABAergic neurons and gives rise to more mesoaccumbal projections than does the rostral VTA (Swanson, 1982). This area is also known to be more important for the rewarding and addictive effects of drugs of abuse than the rostral VTA (Ikemoto, 2007; Krebs *et al*, 2011).

Recording of Neuronal Activity

Extracellular electrical activity was recorded using a newly developed wireless recording system (TeleSpike; Alpha

Omega GmbH, Israel). The transmitter was placed on the animal's back using a special jacket and was connected to the pre-implanted MEA via an Omnetics connector. The receiver was connected to the computer via a USB cable.

Monitoring of Locomotor Activity

During the experiment, animals were placed into $30 \times 43.2 \times 43.2\,cm^3$ Plexiglas chambers. Their locomotor activity was monitored using an MED-OFA Activity Monitor (MED Associates, St. Albans, VT).

To habituate the animals to the experimental conditions, each of them was placed into the experimental chamber for 30 min 2 days before the surgery and for 1, 2, 3, and 3.5 h, respectively, during the 4 days after the surgery. Experiments were run 4–6 days after surgery.

Experimental Protocols

Non-anesthetized animals ($n_{rats} = 23$) were placed into the experimental chambers 30 min before the beginning of the protocol. The protocol consisted of four consecutive sessions, each 45 min long:

- Session 1: Electrical activity and behavior was recorded continuously.
- Session 2: At the beginning of the session, animals received an injection of saline (0.5 ml, i.p.; $n_{rats} = 23$).
- Session 3: At the beginning of the session, animals received an injection of cocaine (10 mg/kg in 0.5 ml of saline, i.p.; $n_{rats} = 13$) or saline (0.5 ml, i.p.; $n_{rats} = 10$).
- Session 4: At the beginning of the session, animals received an injection of the D2/D3 agonist quinpirole (100 μg/kg, an autoreceptor-selective dose, in 0.5 ml of saline, i.p.; $n_{rats} = 23$).

Animals that were anesthetized with chloral hydrate (400 mg/kg, supplemented as needed; $n_{rats} = 14$) underwent the same experimental protocol. At the beginning of Session 3, seven rats received an injection of cocaine (10 mg/kg in 0.5 ml of saline, i.p.) and the other seven rats received an injection of saline (0.5 ml, i.p.).

The dose of 10 mg/kg cocaine is assumed to be a low-range dose for the i.p. route of administration (Horger *et al*, 1999; O'Dell *et al*, 1996; Porrino, 1993). In addition, we performed the same experiments on awake ($n_{rats} = 5$) and anesthetized ($n_{rats} = 5$) rats, with a 10 times lower dose of cocaine (1 mg/kg, i.p.).

Identification of DA Neurons

Electrophysiological and pharmacological parameters were used in order to identify DA neurons. *In vivo*, these neurons exhibit an irregular firing pattern with interspersed bursting episodes and long (>1.5 ms), triphasic spikes, often displaying a prominent notch in the initial positive rising phase. They have a slow firing rate (range: 0.5–5 Hz; Bunney *et al*, 1973b). In addition, we used a pharmacological control, namely an intraperitoneal injection of the D2/D3 agonist quinpirole (100 μg/kg), at the end of each experiment (see above: Session 4). This dose is supposed to predominantly activate D2/D3 autoreceptors (Li *et al*, 1996; White and Wang, 1983), thereby inhibiting the firing of those DA neurons that have a high density of these receptors on the soma and dendrites, that is, most of midbrain DA neurons, except the mesoprefrontal ones (Chiodo *et al*, 1984; Ford and Williams, 2008; Lammel *et al*, 2008). Units that had broad spikes, a low firing rate, and exhibited a slowing (of at least 50%) or a complete cessation of their firing after quinpirole injection were assumed to be 'non-mesoprefrontal' VTA DA neurons.

We did not use the spike shape as a criterion because the exact electrode position could not be controlled in our experimental conditions. Several studies have demonstrated that the extracellular spike waveform varies with the electrode position relatively to the site of action potential generation in the recorded cell (Berretta *et al*, 2010; Cohen and Miles, 2000; Gold *et al*, 2006).

Data Analysis

The fast transients corresponding to spontaneous action potentials were captured online using the Alpha Omega TeleSpike software, and were subsequently transferred to the Spike2 6.0 software (Cambridge Electronic Design, Cambridge, UK) and analyzed off-line by means of an amplitude threshold adjusted by visual inspection in each individual active channel. We also calculated the percentage of spikes generated in a burst firing pattern with regard to the total number of spikes. Bursts were identified using previously established criteria for discriminating burst from non-burst events in A10 and A9 DA neurons (Clark and Chiodo, 1988; Grace and Bunney, 1984). A spike train was considered to be a burst when it contained at least three successive spikes, with the onset defined as an interspike interval of 80 ms or less and burst termination as an interspike interval of 160 ms or greater.

Pharmacokinetic Study

For the pharmacokinetic study, experimental conditions were set as close as possible to those of the electrophysiological recordings. Awake rats ($n_{rats} = 11$) were placed into experimental boxes 30 min before the beginning of the protocol. Each of them then received an i.p. injection of cocaine (10 mg/kg in 0.5 ml of saline). Rats were killed 10 or 30 min after the injection ($n_{rats} = 5$ and 6, respectively).

The other group of rats ($n_{rats} = 12$) was anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). About 15 min after the animals were injected, they received an injection of cocaine (10 mg/kg in 0.5 ml of saline, i.p.), and were killed 10 or 30 min afterwards ($n_{rats} = 6$ and 6, respectively).

Sampling procedures were adapted from Bowman *et al* (1999). Animals were killed by decapitation. Brain tissue and blood collected from trunk vessels were used to measure cocaine and BZE levels (see Supplementary materials for details).

Drugs and Chemicals

Cocaine hydrochloride for i.p. injection was obtained from Fagron (Waregem, Belgium). Heparin was from LEO Pharma (Lier, Belgium). Quinpirole was obtained from

Tocris (Bristol, UK). All LC-MS grade solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and other chemicals were from Sigma (Steinheim, Germany).

Cocaine and BZE reference materials and internal standards were purchased from LGC Promochem (Molsheim, France). All standards had a degree of purity >99%.

Statistical Analysis

All values are presented as means ± SEM. Comparison of the main groups was performed by a hierarchical analysis of variance (ANOVA) using 1 min periods for single data points (bin size). The neurons were nested within the rats, which were in turn nested within the treatment groups (anesthetized *vs* non-anesthetized subjects). The treatment groups were treated as a between-subject factor, while time points before and after cocaine administration were treated as a within-subject factor. Changes in neuronal activity within each group were analyzed using 5 min bins compared with the last or next to last period before infusion (ANOVA for repeated measures followed by Dunnett's *post hoc* test). Statistical significance was set at $p<0.05$.

Cocaine and BZE plasma and brain concentrations were evaluated by a two-tailed Student's *t*-test.

RESULTS

Behavioral Effects of Cocaine (10 mg/kg, i.p.) and Saline Injections

Injection of 10 mg/kg i.p. cocaine at the beginning of Session 3 ($n_{rats}=13$) was followed by a variable increase in locomotor activity and stereotypic movement over 45 min. The animals were divided into two groups: low cocaine responders (LCRs, $n_{rats}=6$) and high cocaine responders (HCRs, $n_{rats}=7$), using the method of Sabeti *et al* (2002). Briefly, the distance traveled by all the animals over the first 30 min was measured and the values were ranked. Animals whose values were below and equal to or above the median were defined as LCRs and HCRs, respectively (see Supplementary Figure S3).

Injection of saline at the beginning of Session 2 ($n_{rats}=23$) and at the beginning of Session 3 ($n_{rats}=10$) provoked only a short-term increase in locomotor activity, which lasted for ~5 min.

Analysis of locomotor behavior during the baseline period did not reveal significant differences between the LCR and HCR groups.

Characteristics of Recorded Neurons

Recordings were made from a total of 134 units in 28 awake rats and a total of 94 units in 19 anesthetized rats. Using the criteria described in the Materials and methods, we classified 107 units in 24 awake animals and 80 units in 19 anesthetized animals as presumably DA. These units had broad spikes (>1.5 ms) and a slow irregular firing, with intermittent periods of bursts. Their average firing frequency during the baseline period was 1.28 ± 0.21 Hz with 14.3 ± 5.8% of spikes in bursts in awake animals and 1.05 ± 0.12 Hz with 10.2 ± 4.3% of spikes in bursts in anesthetized animals (mean values measured over a 20 min period before any intervention). These values were not statistically different from each other. Injection of an autoreceptor-selective dose of quinpirole (100 μg/kg, i.p.) (Li *et al*, 1996; White and Wang, 1983) at the end of the recording session inhibited firing of these neurons by at least 50% (Supplementary Figure S4).

Effect of 10 mg/kg i.p. Cocaine on the Firing Rate and Bursting of DA Neurons

Injection of saline at the beginning of Session 2 (total $n_{units}=163$; total $n_{rats}=33$) and at the beginning of Session 3 (total $n_{units}=84$; total $n_{rats}=16$) did not induce any significant changes in the activity of DA neurons in any of the groups (Supplementary Figure S5).

Figure 1 shows that the effect of 10 mg/kg cocaine on the firing rate of DA neurons was very different in anesthetized and awake animals. This was confirmed by a hierarchical ANOVA test, which yielded a highly significant group-time interaction for the effect of cocaine in the neurons from awake *vs* anesthetized animals ($F_{64,4160}=2.26$; $p<0.00001$), as well as a significant interaction for cocaine *vs* saline in the awake ($F_{64,4160}=2.25$; $p<0.00001$) and in the anesthetized animals ($F_{64,4160}=1.55$; $p<0.01$).

Injection of 10 mg/kg cocaine in anesthetized animals was followed by a decrease in activity of DA neurons (Figure 1). For the whole population ($n_{units}=27$, $n_{rats}=7$) (Figure 1, inset), the decrease was significant starting from the 15th minute ($p<0.05$, Dunnett's *post hoc* test *vs* 5 min before the drug injection). It was significant starting from the 20th minute ($p<0.05$) when comparing with the last 5 min before the injection. Three units in two rats increased their firing rate within 5 min before the injection. Excluding these three units from the statistical calculations (Figure 1, main panel), the decrease of activity became significant starting from the 25th minute ($p<0.05$ *vs* 5 min before the drug injection) or from the 30th minute ($p<0.05$ *vs* last 5 min before the injection).

When considering the whole population of awake rats, injected with 10 mg/kg cocaine ($n_{units}=52$, $n_{rats}=10$), the activity of DA neurons increased after the injection (Figure 1). The increase became significant starting from the 20th minute ($p<0.01$, Dunnett's *post hoc* test *vs* 5 min before the drug injection).

Cocaine, however, induced various patterns of changes in individual DA neurons. This allowed us to separate several subgroups of neurons in each group of rats.

Anesthetized Rats

In 10 out of 27 DA units, the decrease started at 5 min after the injection of 10 mg/kg cocaine, and the activity remained low up to the end of the observation period (Figures 2a and 3c, d). In 6 other units, a sharp short-term increase in activity was observed within 5 min after the injection. The increase lasted for 10 min and then the activity declined and remained low until the end of the observation period (Figures 2b and 3a). This biphasic effect of cocaine on some DA neurons in anesthetized animals was already described previously (Einhorn *et al*, 1988). The basal activity in these two subgroups of neurons did not differ significantly and

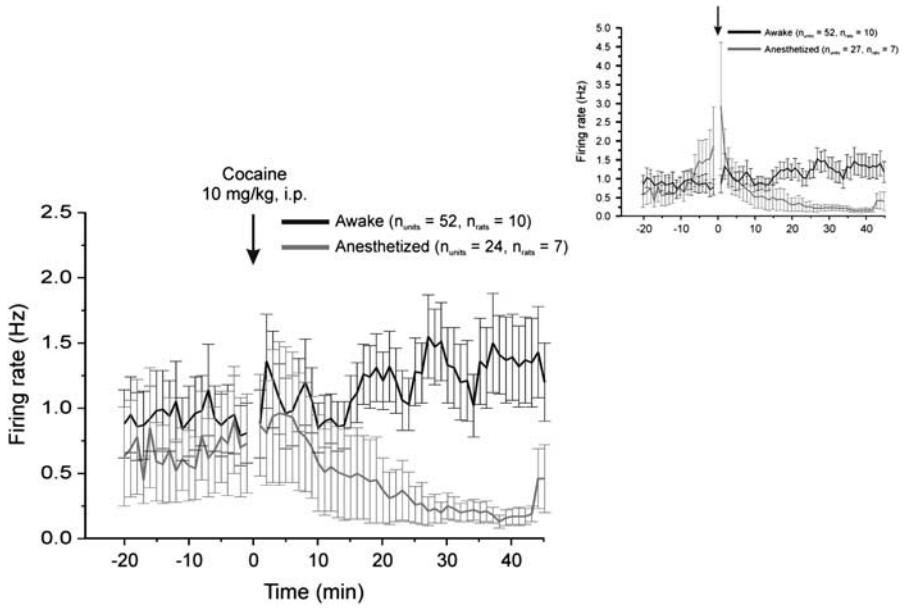

Figure 1 Effect of cocaine (10 mg/kg, intraperitoneally (i.p.)) on the firing rate of dopaminergic (DA) neurons in anesthetized and awake rats. The main panel represents the mean activity of 24 neurons from anesthetized rats. The inset shows the activity from 27 neurons in the anesthetized group. In three of them, an increase in firing rate occurred shortly before the cocaine injection (see text for further details). Error bars represent the standard error of mean (SEM).

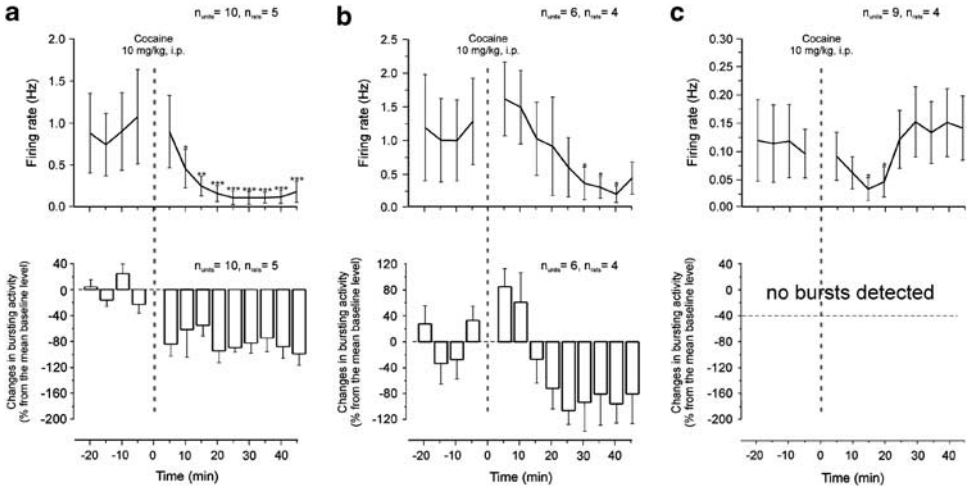

Figure 2 Changes in the firing rate (upper panel) and bursting (lower panel) in dopaminergic (DA) neurons recorded from anesthetized rats after cocaine injection (10 mg/kg, intraperitoneally (i.p.)). The panels show aggregate data from different subgroups (see text). (a) Units that underwent a steady decrease in firing rate immediately after the injection. (b) Units in which a short excitation was seen before the decrease in firing rate. (c) Units that underwent a short-term decrease in firing rate. Error bars represent the standard error of mean (SEM). The degree of significance is represented as $*p<0.05$, $**p<0.01$, $***p<0.001$ (Dunnett's test, comparison to the last 5 min before the injection).

was 1.33 ± 0.14 Hz, with $8.3 \pm 4.8\%$ of spikes in bursts. During the 10 last minutes of the observation period, the firing rate had decreased to 0.23 ± 0.12 Hz with $0.8 \pm 0.6\%$ of spikes in bursts.

In 9 units the activity decreased within 5 min after 10 mg/kg cocaine administration, and recovered 20–25 min after the injection (Figures 2c and 3b). These neurons had low basal firing rate (0.11 ± 0.01 Hz) and did not express any bursting activity.

In two DA neurons, the injection of cocaine was followed by an increase in firing rate from 1.05 and 0.83 to 2.16 and 1.24 Hz, respectively. No bursting activity was detected in these neurons.

Non-Anesthetized Rats

Patterns of changes induced by cocaine in DA neurons from awake rats were much more variable than in anesthetized animals.

In Subgroup 1 (15 units, from 7 animals), injection of cocaine led to a gradual increase in firing rate. The increase lasted up to the end of the 45 min observation period and was significant from the 20th minute ($p<0.05$–0.01, Dunnett's test; Figure 4a). In LCR animals, these units had significantly ($p<0.05$, ANOVA) lower basal firing rates (0.23 ± 0.02 Hz; $n_{\text{units}}=7$, $n_{\text{rats}}=4$) than in HCR animals (1.12 ± 0.07 Hz; $n_{\text{units}}=8$, $n_{\text{rats}}=3$). Firing rates increased

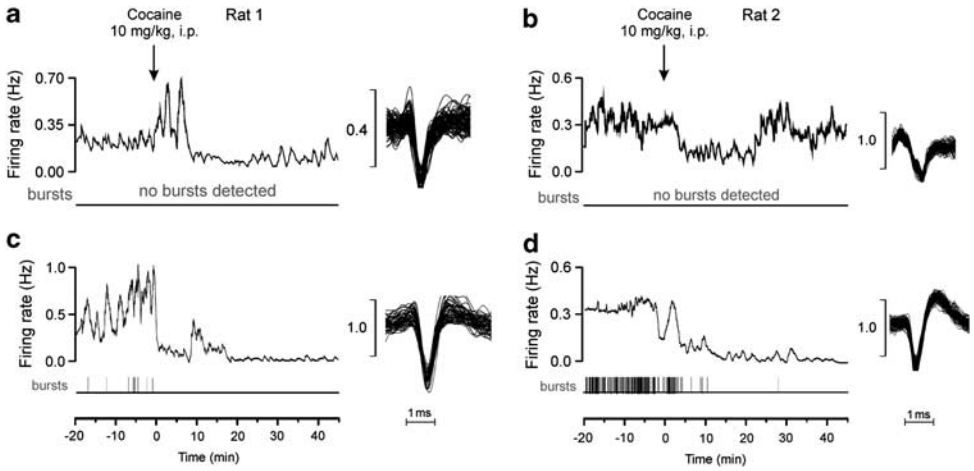

Figure 3 Examples of the effect of a systemic cocaine injection on the firing rate and bursting in two different rats during chloral hydrate anesthesia. Each vertical bar at the bottom of the panels represents an individual burst event (see Materials and methods).

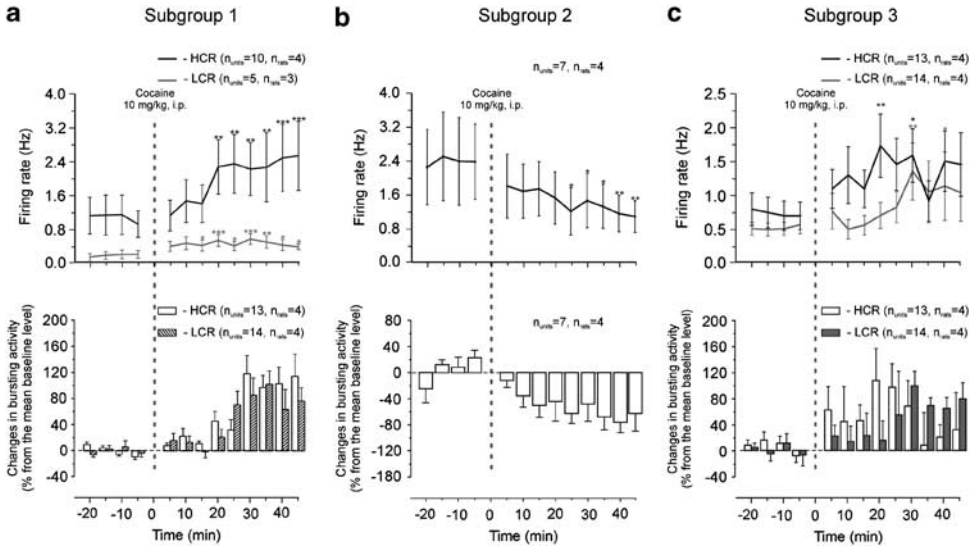

Figure 4 Changes in the firing rate and bursting in dopaminergic (DA) neurons recorded from awake rats after cocaine injection (10 mg/kg, intraperitoneally (i.p.)). (a) Units that underwent an increase in firing rate without correlation to locomotor activity. (b) Units in which a decrease in firing rate was observed. (c) Units in which the increase in firing rate was correlated to locomotor activation. Error bars represent the standard error of mean (SEM). The degree of significance is represented as $*p<0.05$, $**p<0.01$, $***p<0.001$ (Dunnett's test, comparison to the last 5 min before the injection).

up to 0.45 ± 0.05 Hz in LCR rats and up to 2.66 ± 0.09 Hz in HCR rats.

In Subgroup 2 (7 units, from 4 animals), after the injection of cocaine, we observed a significant decrease in activity, similar to the main tendency in anesthetized animals ($p<0.05$–0.01, Dunnett's test from the 20th minute; Figure 4b). Baseline activities of these neurons did not differ significantly in LCR and HCR animals.

Changes in the activity in these two subgroups of neurons did not correlate with the level of the animals' locomotor activity (see examples in Figure 5).

The activity in Subgroup 3 (27 units, from 8 animals) fluctuated. This was the only subgroup of neurons in which the firing rate was positively correlated to the locomotor activity (see Figure 5 for examples). Separate averaging of the activity recorded from HCR and LCR rats showed no significant difference in the basal firing rate of these neurons. However, DA neurons recorded from LCR rats ($n_{\text{units}}=14$, $n_{\text{rats}}=4$) underwent an increase in firing and bursting, which was delayed as compared with those from HCR animals ($n_{\text{units}}=13$, $n_{\text{rats}}=4$), consistent with the slower onset of locomotor activation of the former animals (Figures 4c and 5).

The mean basal firing rate of the neurons in awake rats was different in the three subgroups ($F=7{,}53$, $p<0.01$, hierarchical ANOVA). It was significantly higher in Subgroup 2 (2.32 ± 0.22 Hz with $26.5 \pm 9.3\%$ of spikes in bursts) than in Subgroup 1 (for the whole population, 0.71 ± 0.07 Hz with $5.6 \pm 3.5\%$ of spikes in bursts; $p<0.05$, Dunnett's *post hoc* test), and in Subgroup 3 (for the whole population, 0.64 ± 0.07 Hz with $6.2 \pm 3.2\%$ of spikes in bursts; $p<0.05$, Dunnett's *post hoc* test).

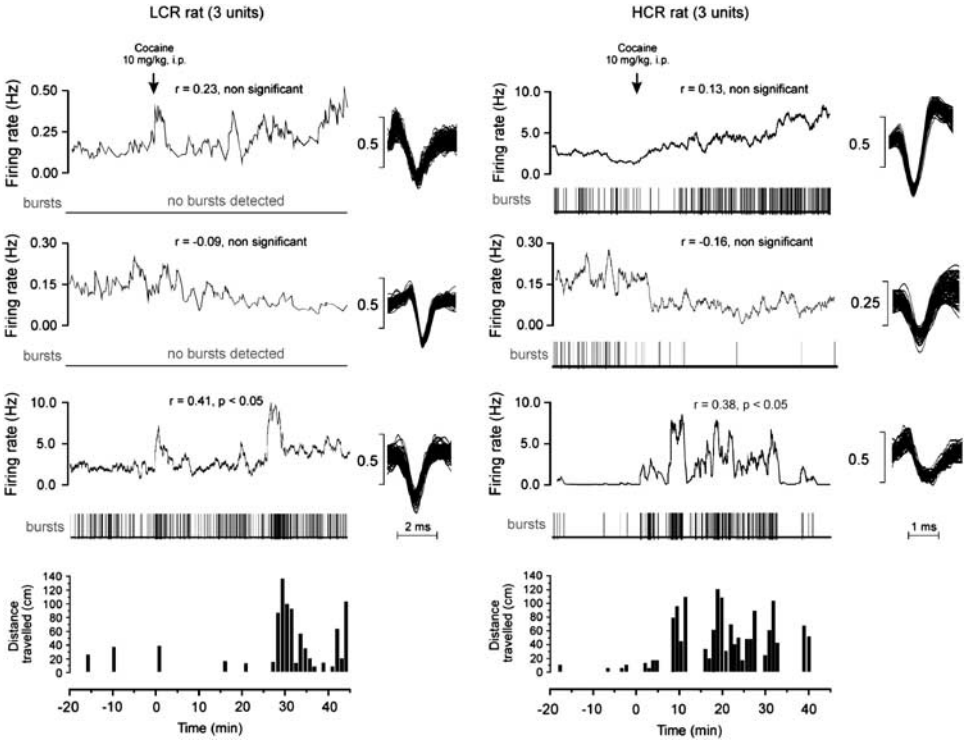

Figure 5 Examples of the effect of cocaine (10 mg/kg, intraperitoneally (i.p.)) on the firing rate, bursting, and locomotor activity in a low cocaine-responsive (LCR) and a high cocaine-responsive (HCR) rat. The degree of correlation between firing rate and locomotor activity (Pearson's coefficient) is indicated for each cell.

The last small subgroup consisted of 3 units (from 2 animals) in which firing rate and bursting decreased within 10 min after the injection of 10 mg/kg cocaine, and returned to the initial level when the locomotor activity of the animals increased at 20–25 min after the drug injection.

Effect of 1 mg/kg i.p. Cocaine on the Behavior and DA Neurons

In anesthetized rats ($n_{\text{units}} = 13$, $n_{\text{rats}} = 5$), i.p. injection of 1 mg/kg cocaine was followed by a slight decrease in firing rate and bursting of DA in two neurons, and a slight increase in firing rate and bursting of DA in one neurons (Supplementary Figure S6A). Other recorded neurons did not show significant changes in activity after the cocaine injection within the period of observation.

In awake animals ($n_{\text{units}} = 11$, $n_{\text{rats}} = 5$), this dose of cocaine led to an increase of activity in one neuron and decrease in another one (Supplementary Figure S6B). Other DA neurons did not undergo significant changes in either firing rate or bursting. These animals also did not show significant changes in the locomotor behavior 45 min after the injection of cocaine. Overall, this dose of i.p. cocaine did not induce any significant electrophysiological effect.

Effect of General Anesthesia on DA Neurons

Injection of chloral hydrate (400 mg/kg, i.p.) in four awake animals during the recording was followed by a fast inhibition of 7 out of 10 DA neurons (3 out of 5, 1 out of 1, 1 out of 2, and 2 out of 2, respectively). The firing rate of three out of these seven neurons was completely inhibited and recovered only by the end of the anesthesia period (see examples in Figure 6).

Results of Pharmacokinetic Study

There were no significant differences in cocaine concentrations between the awake and anesthetized rats in either plasma or brain tissue. Between the 10th and the 30th minute, the concentration of the drug decreased by 50–60% (Figure 7).

BZE concentrations in the plasma were significantly lower in anesthetized animals than in awake rats. Cocaine/BZE ratios in the plasma of anesthetized animals were $>>1$ in contrast to $<<1$ in the awake group. This suggests that breakdown of cocaine was inhibited under chloral hydrate anesthesia, which is in accordance with the finding by other groups that chloral hydrate inhibits cocaine esterase (Benuck *et al*, 1989; Pan *et al*, 1995), the enzyme forming BZE from cocaine (Mets *et al*, 1999).

In the brain, BZE concentrations were also higher in awake animals at 30 min ($p<0.01$, Student's *t*-test) (Figure 7). However, cocaine/BZE ratios in the brain were $>>10$ for both groups, suggesting that different BZE concentrations are unlikely to explain the different effects of the cocaine injection in the two groups.

DISCUSSION

General Findings

The results of this study demonstrate that an acute injection of cocaine differentially affects midbrain DA neurons in awake and anesthetized rats.

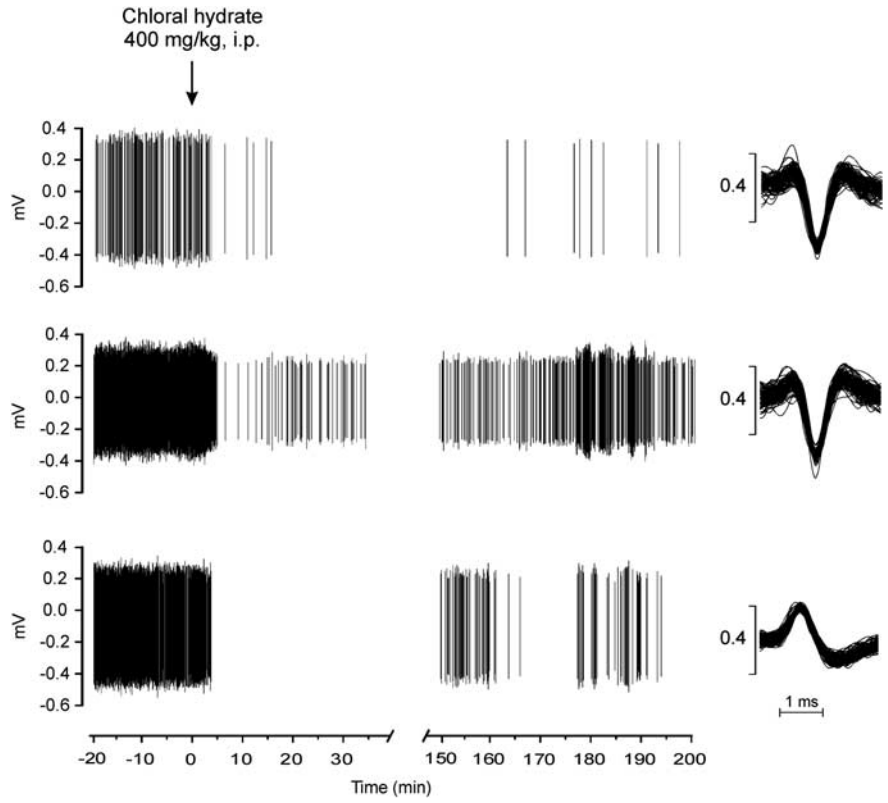

Figure 6 Examples of the effect of chloral hydrate anesthesia (400 mg/kg, intraperitoneally (i.p.)) on the activity of dopaminergic (DA) neurons in one rat.

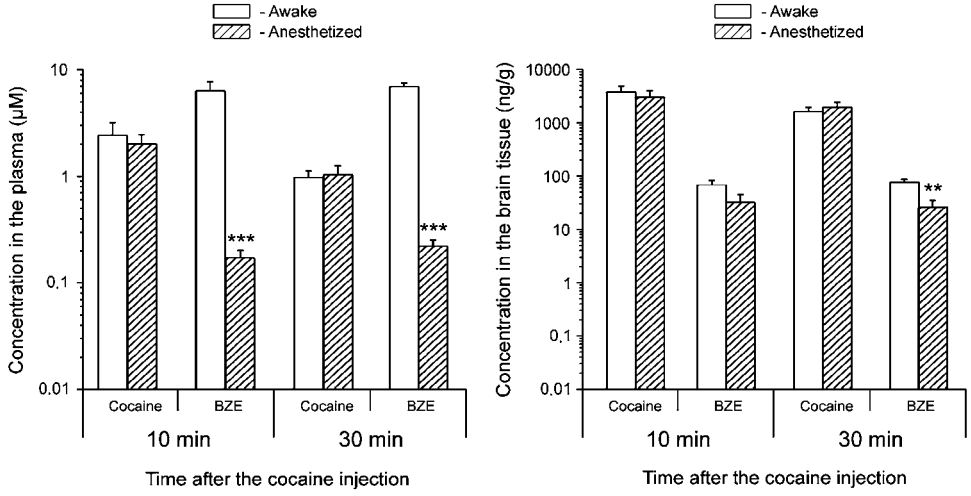

Figure 7 Concentration of cocaine and benzoylecgonine in the plasma and brain tissue of awake and chloral hydrate-anesthetized rats, 10 and 30 min after an intraperitoneal (i.p.) injection of 10 mg/kg cocaine. Results are expressed on a logarithmic scale as means ± standard error of mean (SEM). $^{**}p<0.01$, $^{***}p<0.001$.

In anesthetized rats, the i.p. injection of 10 mg/kg cocaine led to a decrease in firing rate and bursting in most of the recorded neurons. This is an anticipated effect, consistent with the knowledge about autoregulatory mechanisms within the mesolimbic pathways (Kalivas, 1993; Wang, 1981; White and Wang, 1984a, b, 1986) and congruent with the action of cocaine on the dopamine transporter (Kuhar *et al*, 1991). It is consistent with previous electrophysiological experiments conducted *in vivo* under general anesthesia (Einhorn *et al*, 1988; Hinerth *et al*, 2000; Mercuri *et al*, 1992).

By contrast, in non-anesthetized rats, injection of the same dose of cocaine increased the firing rate and bursting of a majority of DA neurons.

As discussed below, a number of mechanisms may account for the excitatory effects of cocaine in awake animals. We will discuss separately the two patterns of excitatory effects.

Excitatory Effect of Cocaine on DA Neurons in Non-Anesthetized Rats

The most intriguing observation of our study was that 29% of DA neurons in the VTA (Subgroup 1) of awake rats underwent a steady increase in firing rate without any significant correlation to locomotor activity. To our knowledge, this effect has never been described before, and it was not observed in anesthetized animals in our hands.

One attractive explanation for this result is the induction of a long-term potentiation of glutamatergic synapses on DA neurons. Such a phenomenon has been observed after a single exposure to cocaine in brain slices (Ungless *et al*, 2001). This potentiation has at least two phases, involving NMDA and AMPA receptors, respectively (Argilli *et al*, 2008). Enhancement of NMDA-mediated transmission can be already measured within a few minutes (Argilli *et al*, 2008; Schilstrom *et al*, 2006), thus having a time course that is compatible with the one of our experiments (Figure 4a), suggesting that these events might have a similar origin. Schilstrom *et al* (2006) found that cocaine-induced NMDA potentiation is due to the activation of protein kinase A via D5 receptors, located on the membrane of VTA DA neurons. It will therefore be interesting in future experiments to test whether co-treatment with a D1/D5 antagonist prevents this pattern of excitation of VTA DA neurons after cocaine injection in awake animals.

It should also be remembered that several VTA-projecting areas release neurotransmitters that are able to excite DA neurons (Geisler *et al*, 2007; Mathon *et al*, 2003; White, 1996). Geisler *et al* (2008) demonstrated that at least some of these afferents are activated in awake animals by repeated cocaine administration, which can in turn lead to the activation of VTA DA cells. General anesthesia can abolish this effect of cocaine, as it markedly decreases the activity of many central neurons. This can explain the difference between awake and anesthetized rats in our experiments.

There are other possible explanations for the stimulating effect of cocaine on the VTA DA neurons. Because it is a local anesthetic (O'Leary and Chahine, 2002), the drug can use-dependently inhibit faster-firing GABAergic neurons in the VTA, thus decreasing the inhibitory input to the DA neurons (Steffensen *et al*, 2008). However, the local anesthetic effect of cocaine may require higher concentrations of the drug in the brain tissue than those achieved in our experiments (Strichartz, 1976). Another possibility is that, by blocking the serotonin transporter (Reith *et al*, 1986; Ritz *et al*, 1990), cocaine may increase the concentration of serotonin in the VTA (Reith *et al*, 1997a), leading to the activation of inhibitory presynaptic $5HT_{1D}$ receptors on GABA terminals (Cameron and Williams, 1994; Johnson *et al*, 1992), and therefore disinhibition of DA neurons.

A final explanation is provided by the block of the noradrenaline transporter by the drug. The concentration of noradrenaline is known to increase after the injection of cocaine (Reith *et al*, 1997a). This could increase the spontaneous firing rate in VTA DA neurons, by activating α_1 receptors (Grenhoff *et al*, 1995).

In 52% (27 out of 52) of DA units (Subgroup 3), both firing rate and bursting fluctuated, with a clear correlation to the locomotor activity of the animals. We do not know whether fluctuations in the activity of these neurons were secondary to the cocaine-induced locomotor effects, or whether changes in their firing underlied locomotor activation. The fact that VTA neurons play an important role in motivation makes the latter interpretation very attractive, but this issue needs further investigation.

Differences Between Rats Classified as HCR or LCR

Behavioral effects of cocaine that were observed in this study are in a good agreement with previous observations in Sprague–Dawley rats (Gulley *et al*, 2003; Mandt and Zahniser, 2010; Sabeti *et al*, 2002).

We would also like to emphasize the difference observed in the basal firing rate of the Subgroup 1 neurons recorded from LCR and HCR rats. In LCR animals, these neurons had lower basal firing rates than in HCR rats (Figure 4a). Similar differences in the basal firing rate were reported for VTA DA neurons between rats expressing a low and high reaction to novelty (McCutcheon *et al*, 2009). Interestingly, animals exhibiting a higher response to novelty also have an enhanced susceptibility to addiction (Belin *et al*, 2009; Cain *et al*, 2004; Grimm and See, 1997; Marinelli and White, 2000; Piazza *et al*, 1989, 1990, 2000; Pierre and Vezina, 1997; Stoffel and Cunningham, 2008). Moreover, the reactivity of their VTA DA neurons is suggested to be critical for the development of addictive behavior (Marinelli *et al*, 2003, 2006; Marinelli and White, 2000; McCutcheon *et al*, 2009; Vezina, 2004; White and Kalivas, 1998a).

Although the relationship between the levels of locomotor response to novelty and of cocaine-induced locomotion remains controversial (Allen *et al*, 2007; Chefer *et al*, 2003; Gulley *et al*, 2003; Hooks *et al*, 1991; Mercuri *et al*, 1989; Sabeti *et al*, 2002), our findings suggest a possible common substratum of these two types of behavior.

Physiological mechanisms underlying lower and higher behavioral reactivity to cocaine are not completely clear. The diversity of locomotor reactivity might be due to a recently demonstrated difference in the levels of DAT activity in the NAc and striatum (Sabeti *et al*, 2002), leading to different extracellular DA levels (Nelson *et al*, 2009).

Possible Role of the Recorded DA Neurons in Reinforcing and Behavioral Effects of Cocaine

DA neurons from the caudal and dorsal VTA send projections to the ventral regions of the striatum, particularly to the NAc (Beckstead *et al*, 1979; Fallon and Moore, 1978; Ikemoto, 2007; Koob, 1992; Pierce and Kumaresan, 2006; Swanson, 1982). DA cells from the lateral portion of VTA mostly project to the ventral pallidum (Klitenick *et al*, 1992). Both NAc and ventral pallidum play a key role in drug addiction (Hubner and Koob, 1990; Koob, 1992; Robinson and Berridge, 2003). We suggest that those DA neurons in the dorsocaudal VTA, which expressed steady activation after 10 mg/kg cocaine injection in our experiments (Figure 4a), represent a part of the drug reinforcement circuit. Further studies are needed to evaluate possible relationship between the difference in the basal firing rate of these neurons observed in LCR and HCR rats and vulnerability of animals to the reinforcing effect of the drug.

At the same time, mesopallidal DA projections play a role in the maintenance of locomotor activity, and

microinjections of DA into the ventral pallidum were shown to stimulate locomotion (Klitenick *et al*, 1992). It is possible that neurons from Subgroup 3 (Figure 4c) in our study belonged to this population.

Effect of General Anesthesia

Differences between drug effects in awake and anesthetized animals have been reported in other brain areas. For example, cocaine increases the activity of striatal and accumbal neurons in freely moving animals (Kiyatkin, 2002; Kiyatkin and Rebec, 2000; Pederson *et al*, 1997; White *et al*, 1998b), but decreases their firing in anesthetized ones (Kreuter *et al*, 2004).

Chloral hydrate anesthesia has often been used in electrophysiological experiments on DA neurons (Einhorn *et al*, 1988; Freeman *et al*, 1989; Grace and Bunney, 1984; Shepard and German, 1988). The reason is that the pattern of activity of DA neurons recorded from chloral hydrate-anesthetized animals is relatively similar to the pattern of activity of DA neurons recorded from animals in the awake state (Freeman and Bunney, 1987; Hyland *et al*, 2002). This led to the assumption that chloral hydrate has a mild effect on the afferent synaptic inputs to the midbrain DA neurons. However, the tone of afferent inputs in anesthetized animals is probably different from the one in behaving animals, as anesthesia changes the activity and sensitivity of different components of central circuitry, including those sending afferent projections to the midbrain DA system (Hamilton *et al*, 1992; Heym *et al*, 1984; Kreuter *et al*, 2004; Trulson and Trulson, 1983a, b; Warenycia and McKenzie, 1984).

Moreover, chloral hydrate does affect midbrain DA neurons themselves, altering their responsiveness to various centrally acting drugs (Bunney *et al*, 1973a; Gessa *et al*, 1985; Kelland *et al*, 1990, 1989; Melis *et al*, 1998; Mereu *et al*, 1987), particularly enhancing the ability of DA agonists to inhibit activity of DA neurons (Kelland *et al*, 1989).

A clear effect of chloral hydrate on DA neurons was observed in our study. Indeed, a majority of the DA neurons recorded in awake rats were partly or fully suppressed by chloral hydrate injection (Figure 6). At the same time, we did not find significant differences in the baseline firing rate of DA neurons between awake and anesthetized animals (Figure 1). Collectively, these observations suggest that populations of DA neurons recorded in the awake and anesthetized animals were different—a large fraction of the DA neurons, contributing to the firing rate under general anesthesia, were silent in the awake state, and *vice versa*. This would explain the difference in the cocaine effects, especially in view of the recent observation that the properties of glutamatergic synapses onto the various subpopulations of VTA DA neurons are heterogeneous (Lammel *et al*, 2011).

Anesthesia also excludes stress, which is known to affect the DA system. In the awake animals, a mild stress could potentiate DA neuron firing, as has been shown for mesoprefrontal neurons (Thierry *et al*, 1976). However, we tried to minimize stress by careful habituation of the rats before the experimental sessions. We therefore do not favor this explanation as a reason for the observed differences.

Lack of Effect of Anesthesia on Brain Cocaine Concentrations

The previously demonstrated ability of general anesthesia to alter cocaine metabolism (Benuck *et al*, 1989; Pan *et al*, 1995) suggested that differences in the cocaine effects between awake and anesthetized rats might have a pharmacokinetic origin. We found the expected difference in plasma BZE concentrations, which is explained by the inhibition of cocaine esterase by chloral hydrate (Benuck *et al*, 1989; Pan *et al*, 1995). However, cocaine concentrations in the brain were not significantly different in the awake and anesthetized animal, and were much higher than the concentration of BZE. These results allow us to suggest that the differences observed in our electrophysiological experiments have a pharmacodynamic rather than a pharmacokinetic origin.

CONCLUSIONS

This study demonstrates strikingly different effects of i.p. injected cocaine on the activity of DA neurons in awake and anesthetized animals. Such a difference is also likely to exist for other drugs of abuse. Telemetric recordings in awake animals may allow investigators to uncover drug effects, which are masked by anesthetics because of a general decrease in neuronal excitability and connectivity. It will be interesting in the future to study possible changes in DA neuron excitability during cocaine reinforcement in self-administration protocols.

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DISCLOSURE

The authors declare no conflict of interest.

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