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ORIGINAL INVESTIGATION

Lack of correlation between the activity of the mesolimbic dopaminergic system and the rewarding properties of pregabalin in mouse

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Abstract

Rationale Pregabalin is a psychoactive drug indicated in the treatment of epilepsy, neuropathic pain, and generalized anxiety disorders. Pregabalin acts on different neurotransmission systems by inactivating the alpha2-delta subunit of voltage-gated calcium channels. In light of this pharmacological property, the hypothesis has been raised that pregabalin may regulate the mesolimbic dopamine pathway and thereby display a potential for misuse or abuse as recently observed in humans. Although some preclinical data support this possibility, the rewarding properties of gabapentinoid are still a matter for debate.

Objective The aim of this work was to evaluate the rewarding properties of pregabalin and to determine its putative mechanism of action in healthy mice.

Results Pregabalin alone (60 mg/kg; s.c.) produced a rewarding effect in the conditioned place preference (CPP) test albeit to a lower extent than cocaine (30 mg/kg; s.c.). Interestingly, when assessing locomotor activity in the CPP, the PGB60 group, similarly to the cocaine group, showed an increased locomotor activity. In vivo single unit extracellular recording showed that pregabalin had mixed effects on dopamine (DA) neuronal activity in the ventral tegmental area since it decreased the activity of 50% of neurons and increased 28.5% of them. In contrast, cocaine decreased 75% of VTA DA neuronal activity whereas none of the neurons were activated. Intracerebal microdialysis was then conducted in awake freely mice to determine to what extent such electrophysiological parameters influence the extracellular DA concentrations ([DA]ext) in the nucleus accumbens. Although pregabalin failed to modify this parameter, cocaine produced a robust increase (800%) in [DA]ext.

Conclusions Collectively, these electrophysiological and neurochemical experiments suggest that the rewarding properties of pregabalin result from a different mode of action than that observed with cocaine. Further experiments are warranted to determine whether such undesirable effects can be potentiated under pathological conditions such as neuropathic pain, mood disorders, or addiction and to identify the key neurotransmitter system involved.

Keywords Rewarding properties · Pregabalin · Dopamine · VTA · Nucleus accumbens

Bruno P. Guiard and Emilie Jouanjus co-directed this study.

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Introduction

The neuroanatomical reward circuitry (Feltenstein and See 2008; Volkow and Morales 2015) involved in addiction underlies the integration of pleasurable stimuli induced by motivational behaviors found in substance use disorders and recruits the dopaminergic mesocorticolimbic neuronal system. Dopaminergic neurons from the ventral tegmental area (VTA) have projections in several cerebral areas including the nucleus accumbens (NAc) (Marie and Noble 2012). The release of dopamine (DA) in the NAc generates positive reinforcement under physiological condition, which motivates to seek behaviors sources of pleasure. Under the addictive state uncontrolled release of DA generates a behavioral response leading to the compulsive seeking of reward. Substances with an abuse/addiction potential activate the reward system and increase dopaminergic transmission in the NAc. For example, amphetamines increase DA release and cocaine inhibits its reuptake (Pettit and Justice 1991). Other substances have indirect action, decreasing the inhibition of dopaminergic transmission physiologically induced in the VTA by GABAergic neurons. For example, the binding of opioids to micro-opioid receptors expressed on GABAergic neurons located in the VTA blocks GABA-induced tonic inhibition of DA neurons and leads to increased extracellular DA concentrations (Matsui et al. 2014).

The pharmacological properties underlying the abuse/addiction potential of several psychoactive substances are currently not precisely established. It is notably the case for pregabalin (PGB). PGB is the active principle of a medical drug (Lyrica® and generic specialties) indicated for use in epilepsy and neuropathic pain, and since 2006 for the treatment of generalized anxious disorders. It is a gabapentinoid, i.e., a structural analog of GABA presenting no effect on GABAergic transmission (Ben-Menachem 2004). In contrast, PGB displays a high affinity toward voltage-gated calcium channel α2-δ1 subunits (Ki = 180 nM) (Li et al. 2011). This subunit is expressed by muscular cells (skeletal, cardiac, smooth) and also found in the central and peripheral (dorsal root ganglion) nervous systems (Dolphin 2013). The cerebral regions expressing PGB binding sites are numerous and notably include the cerebral cortex, the hippocampus, the amygdala, and the cerebellum (Bian et al. 2006). Voltage-gated calcium channels are inhibited by PGB, which prevents neuronal depolarization thereby decreasing the release of various neurotransmitters such as glutamate or substance P (Taylor et al. 2007).

The eventuality of an abuse/addiction potential has been specifically examined in vitro and in vivo during the pharmaceutical development of PGB (Balster and Bigelow 2003). In 2001, a conditioned place preference (CPP) study concluded that PGB did not have rewarding properties in the rat and decreased those of morphine (Andrews et al. 2001). Ten years later, a new study using the same test but a different protocol (route of administration) challenged these results (Rutten et al. 2011). In humans, the reported rate of euphoria among patients who participated in PGB clinical trials was sixfold greater in those treated with PGB than in those treated with placebo (Zaccara et al. 2011). The first PGB-related abuse/addiction cases were reported shortly after PGB was marketed in European countries and in the USA (Olaizola et al. 2006; Yargic and Ozdemiroglu 2011; Filipetto et al. 2010; Papazisis et al. 2013). Although inaugural addiction to PGB without history of substance abuse is described (Driot et al. 2016a), these cases most frequently occur in subjects of male gender and in a context of polydrug use or with a history of substance use disorders (Gahr et al. 2013; Schjerning et al. 2016). Several PGB-related abuse/addiction alerts have been identified from national pharmacovigilance system databases (Schwan et al. 2010; Bossard et al. 2016). On the Internet, PGB is described by users who divert it for recreational purposes as "an ideal psychotropic drug" with anxiolytic effects mixed with euphoria (Schifano et al. 2011). In addition, the number of patients self-administering higher than recommended doses of PGB to achieve euphoric highs has increased, and the prevalence of gabapentinoid abuse has at least doubled among opioid abusers (Evoy et al. 2017). By contrast, another review of literature concluded that the rewarding properties of PGB, although higher than those of gabapentin, were low and occurred only in doses assumed to be supratherapeutic (Bonnet and Scherbaum 2017).

So, there are still questions on the mechanism underlying PGB's abuse/addiction potential. In 2003 already—i.e., at a time when PGB was in stage III clinical trials—a review article on GABAergic drugs presented the potential wide therapeutic applications of PGB but noted the absence of available data on long-term use and thus, concluded with the need to evaluate tolerance, dependence and withdrawal or discontinuation reactions (Ashton and Young 2003). On this background, the present study was aimed at comparing the effects of PGB and cocaine in healthy mice to gain insight into the mechanisms controlling the effects of this pharmacological agent observed in human. Assuming that PGB's neuromodulatory effects on the meso-accumbens DA neuronal system could be at the origin of the related abuse/addiction, we combined behavioral, electrophysiological, and neurochemical approaches to gain valuable insight into its pharmacological properties.

Experimental procedures

Animals

Adult C57BL/6j male mice were purchased from the Janvier Laboratories (Le Genest St. Isle, France). Animals were housed four to five per cage with a 12/12 h day/night cycle

(lights on at 7:00 am) in temperature-controlled rooms (22° ± 2 °C). Food and water were available ad libitum. After arrival, animals were allowed to acclimatize for at least 2 weeks before testing during the light phase of the day-night cycle. All experiments were carried out in accordance with the European guidelines for the care of laboratory animals (European Communities Council Directive 86/609/ECC) and approved by the local Ethics Committee and the French Ministry of Education and Research (APAFIS#6659-2016090512114815 v3).

Drugs

All pharmacological drugs were dissolved in 0.9% saline and injected using the subcutaneous (s.c.) route which represents a rapid, inexpensive, and simple method of systemic substance administration with a good absorption rate (Kagan 2014). For acute experiments, we tested pregabalin (PGB, Manchester Organics Ltd., UK) at the increasing doses of 10, 30, and 60 mg/kg. The choice of these doses was based on previous observations that an oral administration of PGB (3–30 mg/kg) had no effect in the CPP test (Andrews et al. 2001). Cocaine (Sigma-Aldrich, Saint-Quentin Fallavier, France) was tested at the dose of 30 mg/kg and used herein as a positive control. For chronic experiments, repeated administrations of PGB (60 mg/kg) and cocaine (30 mg/kg) for 4 days were performed. Pharmacological controls for the electrophysiology experiments were conducted using apomorphine (0.5–1 mg/kg, s.c.) and haloperidol (5 mg/kg, s.c.), a D2 receptor agonist and antagonist, respectively (Sigma-Aldrich, Saint-Quentin Fallavier, France). For all administrations, the volume of injection was 10 mL/kg.

Conditioned place preference

CPP test was done in a custom-made plastic apparatus consisting of two equal size chambers (25 × 25 × 25 cm). A closed door separated these chambers during the conditioning days whereas the animals had free access to both compartments during the pre-conditioning and testing days. The two conditioning compartments had different visual and tactile cues. The first compartment contained a rough floor and checked walls (black and white) whereas the second compartment contained a smooth floor with gray plain walls. On the pre-test session, animals were given free access to the two chambers and allowed explore the apparatus for 10 min (familiarization). Animals were then placed a second time in the apparatus 1 h later to freely explore for 20 min. During the latter session, video tracking (Ethovision Noldus, Wageningen, the Netherlands) allowed recording the time spent by the mice in each chamber and their locomotor activity. Animals showing a preference (> 65%) for any of the two conditioning compartments were discarded from the study ($n = 2$). Drug injections were paired at random with a compartment (CS+ chamber), and saline was given in the other side (CS− chamber). The conditioning phase consisted in 4 consecutive training days with two daily training sessions lasting 20 min. Cocaine and PGB were given just prior to placing the animal in the CS+ chamber and saline prior to the CS− chamber (chambers and time of day were counterbalanced within groups). To prevent an association between injection and the time of day, drug injections were counterbalanced between morning and afternoon each day of conditioning period. Moreover, to obtain an effective association between substances tested and a single compartment, the two daily sessions were spaced by 5 h. The day after the final training session, a 20-min test session consisted in giving mice free access to the apparatus in a drug-free state. Animals were initially placed in the compartment opposite to the one in which they started the pre-conditioning session. CPP score was expressed as the percentage of time spent in the CS+ compartment.

Open field

Mice were tested in an open field to assess locomotor activity 20 min after a single subcutaneous administration of saline, cocaine, or PGB 60 mg/kg. The circular field dimensions were 40 × 30 cm. The tests were conducted in the morning, following habituation to the experimentation room (1 h in the home cage). Mice were tracked for 20 min by video tracking (Ethovision Noldus, Wageningen, the Netherlands). Just after drug administration, each animal was placed in the center of the open field at the beginning of the test. The open field was cleaned with 30% ethanol between animals. Locomotor activity score was expressed as distance traveled (in cm).

Study of dopaminergic system activity

In vivo single-unit recordings

Mice were anesthetized with chloral hydrate and placed in a stereotaxic frame (David Kopf mouse adaptor) with the skull positioned horizontally. A glass micropipette (Stoelting, UK) filled with 2 M NaCl was used for recordings. These micropipettes were previously stretched using a gravitational strainer (Narishige, Japan) in order to obtain a resistance between 5 and 8 MΩ. They were then inserted into the ventral tegmental area (VTA) using the following coordinates (in mm from bregma according to the atlas of Paxinos and Franklin 2001): AP − 3 to − 3.3, L ± 0.3 to 0.6, and V 4.1 to 4.6 mm ventral to the brain surface.

The extracellular potential was recorded with an amplifier and filter (300/0.5 Hz). Single-neuron spikes were collected online (CED 1401, SPIKE 2, Cambridge Electronic Design). Specific electrophysiological criteria were used to identify

putative DA neurons. These included long-duration (> 2.0 ms) biphasic or triphasic action potential, with an initial phase exceeding > 1.0 ms in length, a low firing rate (< 10 Hz), and a firing pattern that consisted of irregular single-spike pattern with slow bursting activity (characterized by spike-amplitude decrement) according to Grace and Bunney 1983. The mode of discharge was analyzed by spike interval burst analysis. The onset of a burst was defined as the occurrence of two spikes with an inter-spike interval shorter than 0.08 s. The termination of bursts was defined as an inter-spike interval (ISI) of > 0.16 s.

For acute experiments, each recording lasted at least 20 min. After stabilization, the basal firing rate of VTA DA neurons was measured for 2 min. Saline or pharmacological agents were then injected subcutaneously and a period of at least 5 min was observed between each injection. Again, after stabilization, the firing rate was measured in the last 2 min. This procedure allowed comparing the discharge frequency before and after the systemic administration of saline or the drugs and conduct pairwise comparison for each animal. At the end of each recording, a pharmacological validation was realized using the D2 receptor agonist apomorphine, which was previously shown to reduce VTA DA neuronal activity (Oosterhof et al. 2014). The ability of the D2 receptor antagonist haloperidol to reverse the effects of apomorphine further confirmed the dopaminergic nature of the recorded neurons. Spike2 software was used during the recording to measure the dopaminergic neurons firing rate (Hertz: number of AP/s), burst frequency, and the action potential number into a burst.

For chronic experiments, the basal firing rate of each putative VTA DA neurons encountered per tract was measured for 5 min in PGB or saline-injected mice. Three tracts per animals were conducted in order to determine the average of firing rate of all recorded neurons but also their mode of discharge (burst and spikes per burst).

Intracerebral microdialysis in awake freely moving mice

Extracellular levels of DA were measured in the nucleus accumbens (NAc) of awake, freely moving mice by microdialysis prior to and following drug administration. Samples collections were preceded by a surgical procedure during which the microdialysis probes were inserted into the brain area of interest. Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and implanted with probes in the NAc using the following coordinates (in mm from bregma, AP + 0.98, L ± 1.3, V − 5). Animals were then allowed to recover from the surgery overnight as previously described (Maskos et al. 2005; Barik et al. 2010). This short delay allows the optimization of samples collection by limiting the impairment of dialysis membrane and thereby avoids biased estimates of the neurotransmitter concentrations at the sampling site. Hence, approximately 20 h after surgery, the probes (without guide cannula) were continuously perfused with artificial cerebrospinal fluid (aCSF, NaCl 147 mmol/L, KCl 2.7 mmol/L, $CaCl_2$ 1.26 mmol/L, $MgCl_2$ 1 mmol/L, NaH_2PO_4 2.0 mmol/L, $NaHCO_3$ 2.0 mmol/L; pH 7.4 ± 0.2) at a flow rate of 1.5 μL/min using a CMA/100 pump (Carnegie Medicin, Stockholm, Sweden). Before microdialysis sessions, all animals were carefully checked and if signs of pain were detected in the home cage, experiments were immediately stopped. Otherwise, 2 h after the start of a continuous aCSF perfusion (stabilization period), four fractions were collected every 15 min into small Eppendorf tubes (Eppendorf, Le Pecq, France). These samples were used to measure the basal values of extracellular DA levels (mean ± SEM) calculated for each mouse prior to acute administration of vehicle, cocaine, or PGB. Twelve subsequent 15-min samples were collected (t15–t120) and extracellular DA levels determined as percentage of baseline.

Dialysate samples were analyzed for DA by a high-performance liquid chromatography method (limit of sensitivity near 0.5 fmol/sample, signal-to-noise ratio = 2) using the Chromeleon Lab 7.2 software (Chromeleon Chromatography Data System, Thermo Fisher Scientific, USA).

Data analysis

Results were expressed as mean ± SEM. For electrophysiological and microdialysis (AUC) experiments, unpaired *t* test was used to compare the firing parameters of VTA DA neurons between cocaine-injected mice and their controls. A one-way analysis of variance (ANOVA) for treatment factor was applied to compare these parameters between PGB-injected mice and their controls. For the analysis of the cocaine or PGB effects on DA extracellular levels (kinetics) and the CPP test, a two-way ANOVA with time and treatment factors was used. When a main effect of time or interaction time × treatment was significant, comparisons between treatments were analyzed using a PLSD post hoc test. Finally, for behavior (CPP), the percentage of time spent in the CS+ compartment was analyzed using a one sample *t* test (comparison to the 50% chance value) to reveal a place preference/aversion. Prism7 software (Graphpad, San Diego, USA) was used for these statistical analyses and significance level was set at $p < 0.05$.

Results

Behavioral tests

Conditioned place preference

Mice were subjected to conditioned place preference, thus assessing the rewarding effect of cocaine and PGB. A significant preference was demonstrated by a percentage

of time spent in the conditioning compartment superior to 50% (chance level). After cocaine conditioning (30 mg/kg, s.c.), the place preference for the drug-paired side was statistically significant (66.3 ± 2%, $p = 0.001$; Fig. 1a). For PGB conditioning, only the dose of 60 mg/kg induced a significant preference for the drug-paired side (55.4 ± 1.6%, $p = 0.006$; Fig. 1a). Moreover, a two-way ANOVA (treatment × conditioning) was carried out showing a significant effect of treatment factor ($F_{(3,30)} = 3.04$, $p < 0.04$), conditioning factor ($F_{(1,30)} = 36.3$, $p < 0.001$), and treatment × interaction ($F_{(3,30)} = 3.29$, $p = 0.02$). Post hoc analyses revealed a significant increase of time spent in the drug-paired side after cocaine ($p < 0.001$; Fig. 1) and PGB 60 mg/kg ($p < 0.016$, Fig. 1a) conditioning compared to the pre-conditioning session.

Regarding animal's activity during pre- and post-conditioning tests, a two-way ANOVA (treatment × conditioning) was carried out showing no significant effect of treatment factor ($F_{(3,30)} = 0.37$, $p > 0.05$) but a significant effect of conditioning factor ($F_{(1,30)} = 26.4$, $p < 0.001$). Interestingly, the Bonferroni post hoc test revealed an increase in the distance traveled after 4 days of conditioning with both cocaine and PGB 60 mg/kg ($p < 0.01$ and $p < 0.001$; Fig. 1b).

Locomotor activity

We also monitored the total ambulatory distance traveled in an open field for 20 min following acute drug administration. One-way ANOVA revealed a significant effect of treatment factor ($F_{(2,12)} = 27.8$, $p < 0.001$). We observed that cocaine produced psychostimulant effects (cocaine 20165 ± 2595 vs. NaCl 5685 ± 781 cm, $p < 0.001$) whereas PGB 60 failed to do so (PGB60 5436 ± 558 vs. NaCl 5685 ± 781 cm, $p = 0.99$).

In vivo recordings of VTA DA neurons

Saline (NaCl 0.9%)

In order to verify the stability of VTA DA neuronal activity, we injected saline, and recordings were continued over 15 min. As expected, the electrophysiological properties of VTA DA neurons were not altered in response to saline injection (Fig. 2).

Cocaine (30 mg/kg, s.c.)

Eight DA neurons were identified and recorded within the VTA. The acute administration of cocaine (30 mg/kg, s.c.) decreased the firing rate of 75% of dopaminergic neurons ($n = 6$) whereas it increased this parameter in one cell (12.5%) and had no effect on another one (12.5%). Among the 75% of VTA DA neurons whose discharge was reduced, the mean basal firing rate was 5.4 ± 0.5 Hz before drug injection and it was significantly reduced to 4.5 ± 0.3 Hz ($p = 0.02$; Fig. 3a–d). As depicted in Fig. 3 d, the addition of the D2 receptor agonist apomorphine completely silenced VTA DA neuronal activity, whereas this effect was reversed by the D2 receptor antagonist haloperidol thereby confirming the dopaminergic nature of the recorded neurons. The analysis of the mode of firing showed that the decrease in VTA DA neuronal activity was due to a decrease in the burst frequency ($p = 0.03$; Fig. 3b) but not in the number of spikes per burst ($p = 0.14$; Fig. 3d).

Pregabalin (PGB 10, 30, and 60 mg/kg, s.c.)

Fourteen DA neurons were identified and recorded within the VTA. The acute administration of cumulative doses of PGB (10, 30, and 60 mg/kg, s.c.) produced mixed results. Although

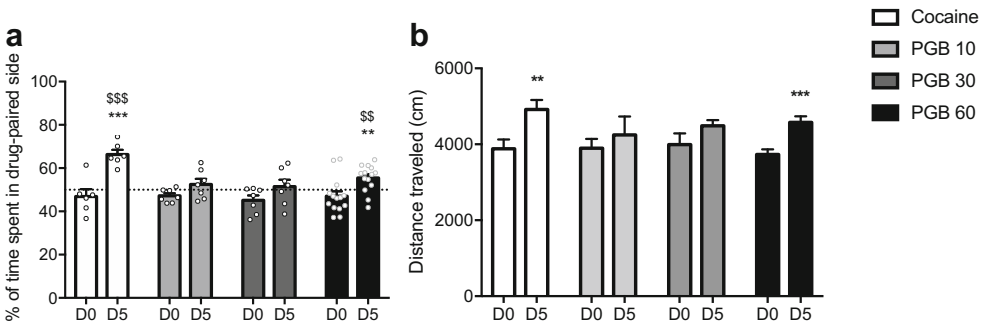

Fig. 1 Effect of cocaine and pregabalin on the development of conditioned place preference. Mean ± SEM of percentage of time spent in drug-paired side after cocaine 30 mg/kg (white, $n = 5$), pregabalin (PGB) 10 mg/kg (light gray, $n = 7$), 30 mg/kg (dark gray, $n = 7$), 60 mg/kg (black, $n = 14$). D0 represents results obtained during the pre-conditioning (dot pattern) and D5 represents results obtained after conditioning for the different groups (unified plain colors) (**a**). $ $p < 0.01$ and $ $p < 0.001$: significantly different from the chance level set at 50%. ** $p < 0.01$ and *** $p < 0.001$: significantly different from the percentage of time spent in the compartment during the pre-conditioning session. As mentioned in the methods, three mice were discarded owing to their spontaneous preference (> 65%) for one compartment before conditioning. Mean ± SEM of distance traveled (in cm) in the absence of drugs in the conditioned place preference apparatus before (D0) and after (D5) conditioning (**b**). ** $p < 0.01$ and *** $p < 0.001$: significantly different from the distance traveled in the arena during the pre-conditioning session (D0)

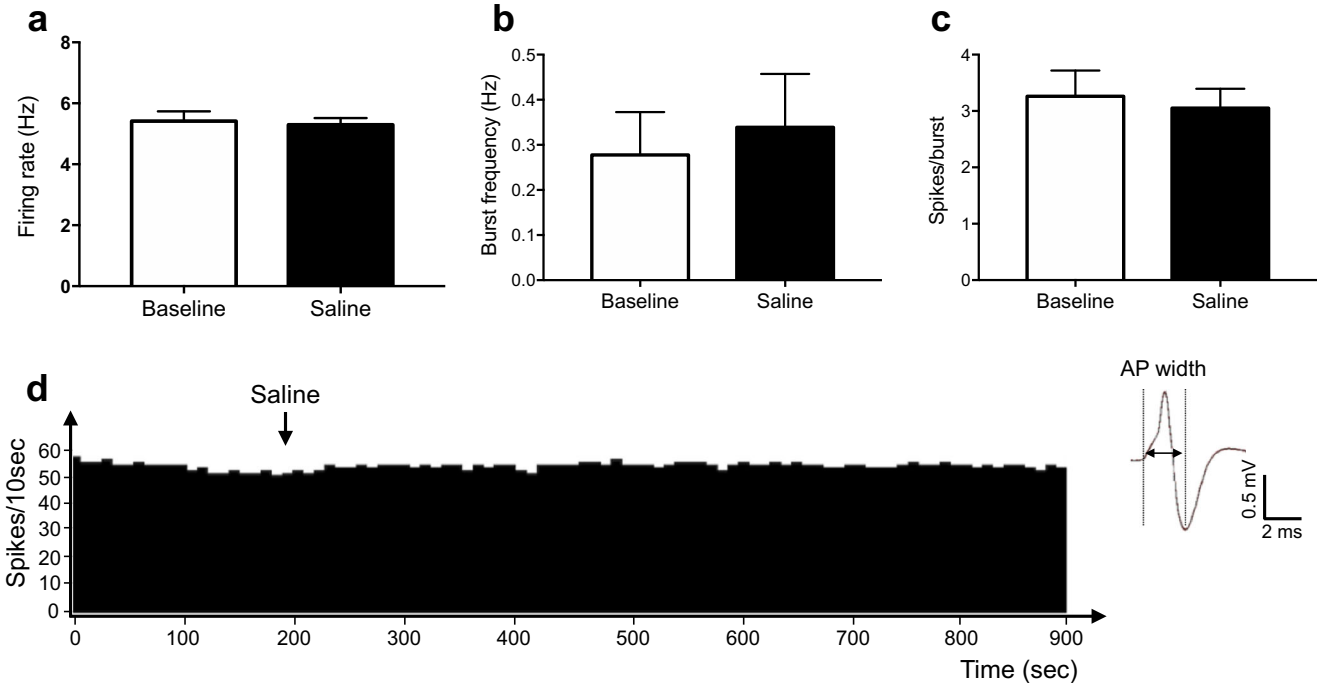

Fig. 2 Lack of inhibition of the VTA dopaminergic neurons firing rate in response to an acute administration of saline. Mean ± SEM of firing rate (Hz) (**a**), burst frequency (Hz) (**b**), and number of spikes per burst (**c**) before (white) and after saline (NaCl 0.9%, s.c.) injection (black) ($n = 6$). **d** Example of integrated firing rate histograms of a VTA dopaminergic neuron showing its stability over time and average extracellular waveform of DA neuron. AP widths were measured from the start of the action potential to the negative trough (top) and used as a criterion to select presumed DA neurons

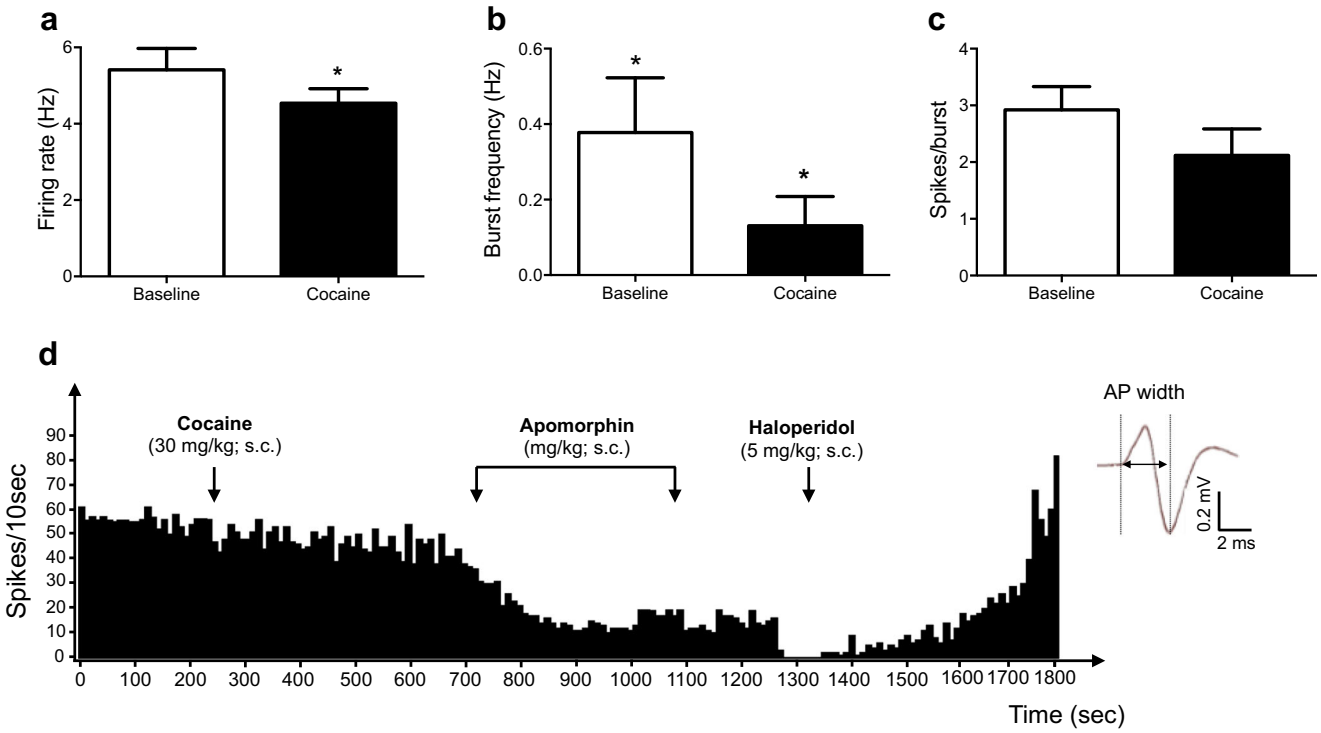

Fig. 3 Inhibition of the VTA dopaminergic neurons firing rate in response to an acute administration of cocaine. Mean ± SEM of firing rate (Hz) (**a**), burst frequency (Hz) (**b**), and number of spikes per burst (**c**) before (white) and after cocaine (30 mg/kg, s.c.) injection (black) ($n = 6$). *$p < 0.05$: significantly different from baseline. Example of integrated firing rate histograms of a VTA dopaminergic neuron showing the responsiveness to injection of cocaine followed by apomorphine (1 mg/kg, s.c.), a D2 receptor agonist and haloperidol (5 mg/kg, s.c.), a D2 receptor antagonist (**d**). Apomorphine injection leads to a full inhibition of the firing rate while haloperidol completely reversed this electrophysiological response. Typical example of average extracellular waveform of DA neuron. AP widths were measured from the start of the action potential to the negative trough (top) and used as a criterion to select presumed DA neurons

PGB 10 and 30 mg/kg failed to modify VTA DA neuronal activity, the highest dose of PGB decreased the firing rate of 50% of VTA DA neurons ($n = 7$), increased 28.5% of them ($n = 4$) while 21.5% did not show any modification ($n = 3$).

Among the 50% of VTA DA neurons whose discharge was reduced, the mean basal firing rate was 5.9 ± 0.6 Hz before drug injection and this parameter was significantly reduced to 4.5 ± 0.5 Hz ($p = 0.01$; Fig. 4a–d). This decrease was not related to an attenuation of the burst frequency and the number of spikes per burst, ($p > 0.05$; Fig. 4b–c). Among PGB-excited VTA DA neurons, ($p = 0.04$; Fig. 5a–d) the mean basal firing rate was 4.4 ± 0.7 before PGB and significantly increased to 5.4 ± 0.9 after drug administration. However, no differences were detected in the burst frequency and the number of spikes per burst ($p > 0.05$).

Microdialysis

Cocaine (30 mg/kg, s.c.)

Two-way ANOVA (treatment × time) on the [DA]ext in the NAc indicated significant main effects of treatment factor ($F_{(1,10)} = 125$, $p < 0.0001$), time factor ($F_{(8,80)} = 37$, $p < 0.0001$) and treatment × time interaction ($F_{(8,80)} = 29.3$, $p < 0.0001$). Cocaine (30 mg/kg, s.c.) administration led to a significant increase in [DA]ext between t15 to t105 with a maximal effect observed around 800% at t30 (Fig. 6a). Statistical analysis on AUC (t0–t120 min) values also indicated an overall significant increase in [DA]ext in the NAc in cocaine-relative to saline-injected mice ($p < 0.011$; Fig. 5b).

Pregabalin (PGB 10, 30, and 60 mg/kg, s.c.)

Two-way ANOVA (treatment × time) on the [DA]ext in the NAc failed to unveil significant main effects of treatment factor ($F_{(3,24)} = 1.964$, $p = 0.14$), time factor ($F_{(8,192)} = 0.7769$, $p = 0.62$), and their interaction ($F_{(24,192)} = 0.9995$, $p = 0.46$). Similarly, statistical analysis on AUC (t0–t120 min) values did not show any difference between treatments ($F_{(3,21)} = 1.968$, $p = 0.15$; Fig. 7a–b) thereby suggesting that PGB had no effect on DA tone in the NAc.

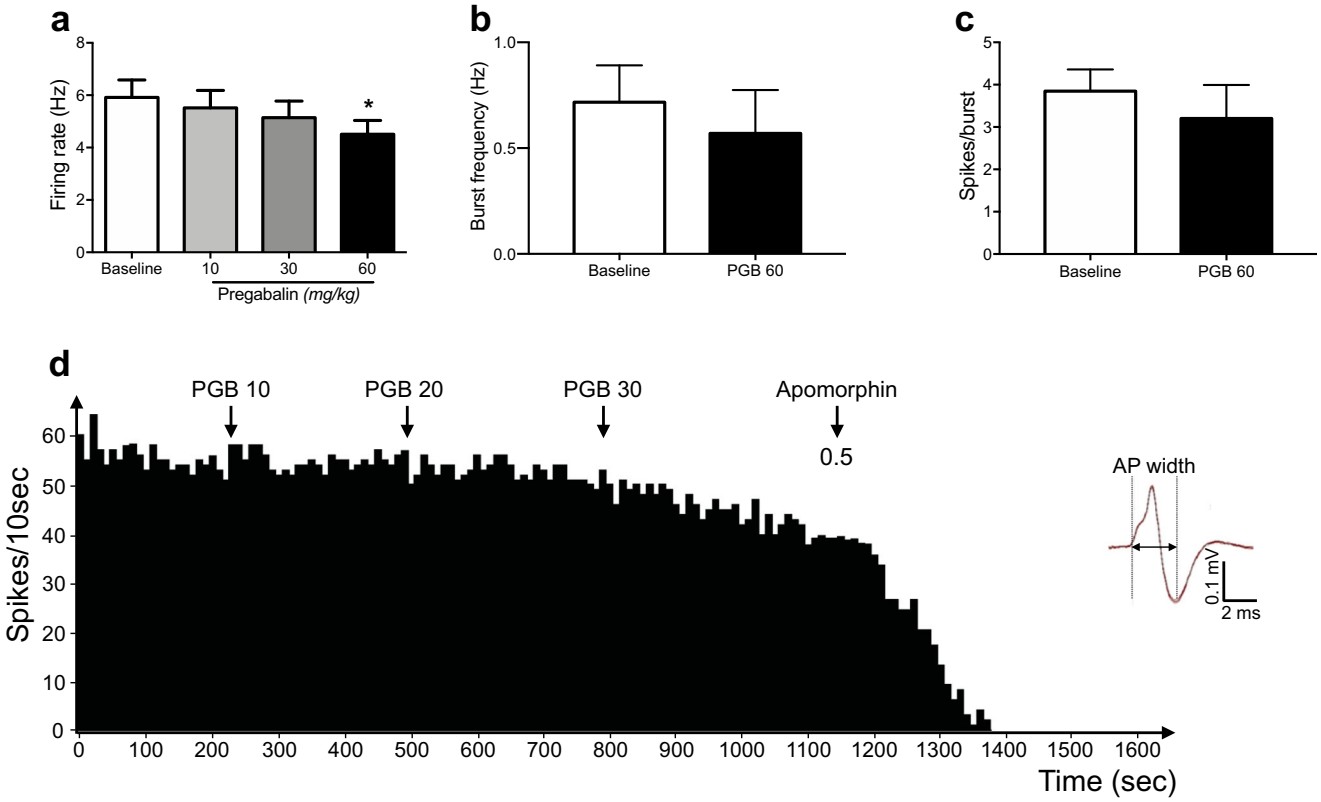

Fig. 4 Inhibition of VTA dopaminergic neurons firing rate in response to an acute administration of pregabalin. Mean ± SEM of firing rate (Hz) (**a**), burst frequency (Hz) (**b**), and number of spikes per burst (**c**) before (white) and after acute repeated administration of pregabalin (PGB) at the doses of 10, 20, and 30 mg/kg, s.c. (gray/black) ($n = 7$). This sequence of administration corresponds to cumulative doses of 10, 30, and 60 mg/kg of PGB. *$p < 0.05$: significantly different from baseline. Example of integrated firing rate histograms of a VTA dopaminergic neuron showing the responsiveness to injection of cumulative doses of PGB followed by an injection of apomorphine (0.5 mg/kg, s.c.) and average extracellular waveform of DA neuron (**d**). AP widths were measured from the start of the action potential to the negative trough (top) and used as a criterion to select presumed DA neurons

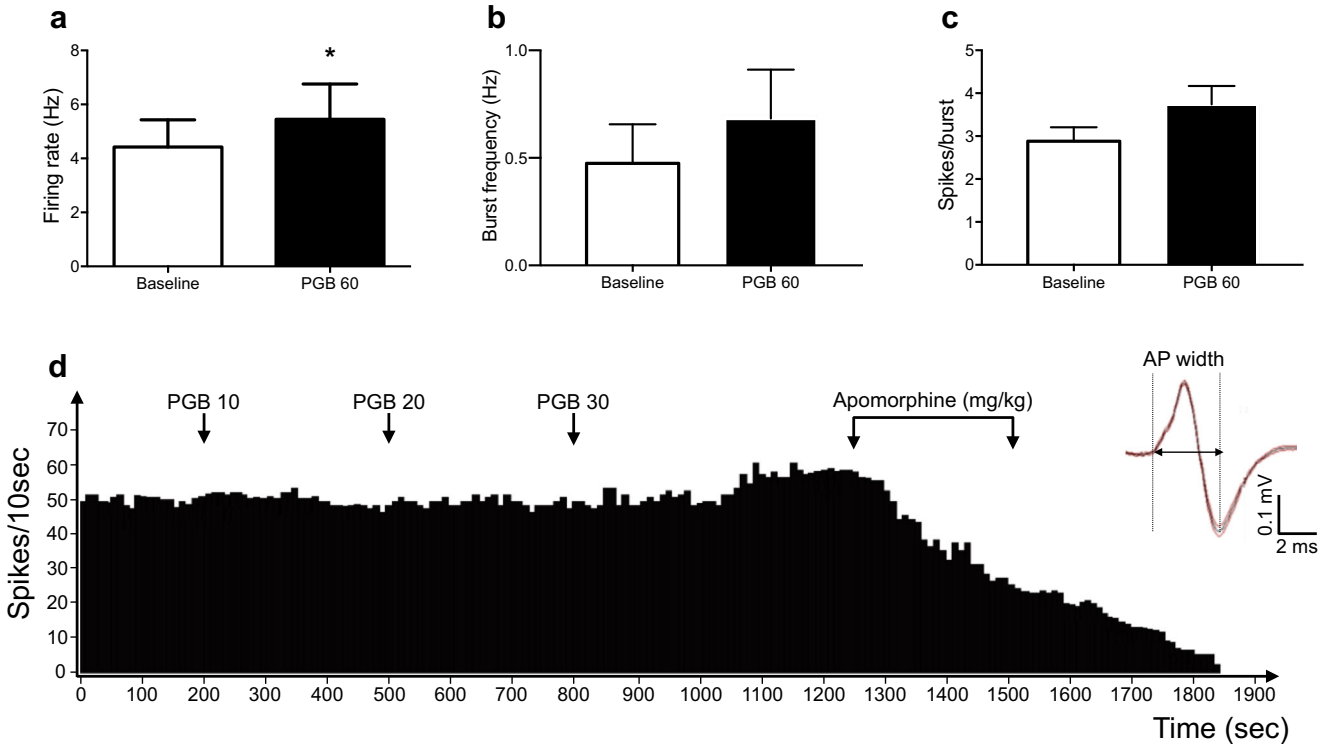

Fig. 5 Excitation of VTA dopaminergic neurons firing rate in response to an acute administration of pregabalin. Mean ± SEM of firing rate (Hz) (**a**), burst frequency (Hz) (**b**), and number of spikes per burst (**c**) before (white) and after acute repeated administration of pregabalin (PGB) at the doses of 10, 20, and 30 mg/kg, s.c. (gray/black) ($n = 4$). This sequence of administration corresponds to cumulative doses of 10, 30, and 60 mg/kg of PGB. *$p < 0.05$: significantly different from baseline. Example of integrated firing rate histograms of a VTA dopaminergic neuron showing the responsiveness to injection of cumulative doses of PGB followed by an injection of apomorphine (0.5 mg/kg, s.c.) and average extracellular waveform of DA neuron (**d**). AP widths were measured from the start of the action potential to the negative trough (top) and used as a criterion to select presumed DA neurons

Repeated administration of pregabalin 60 mg/kg

To study the electrophysiological and neurochemical effects of PGB in the same conditions than those used for behavioral experiments, we evaluated the impact of this pharmacological agent after its repeated administration for 4 consecutive days (Fig. 8a).

Unlike acute administration, repeated injections of PGB (60 mg/kg, s.c.) did no longer inhibit the mean firing rate of dopaminergic neurons and consequently, the neuronal activity

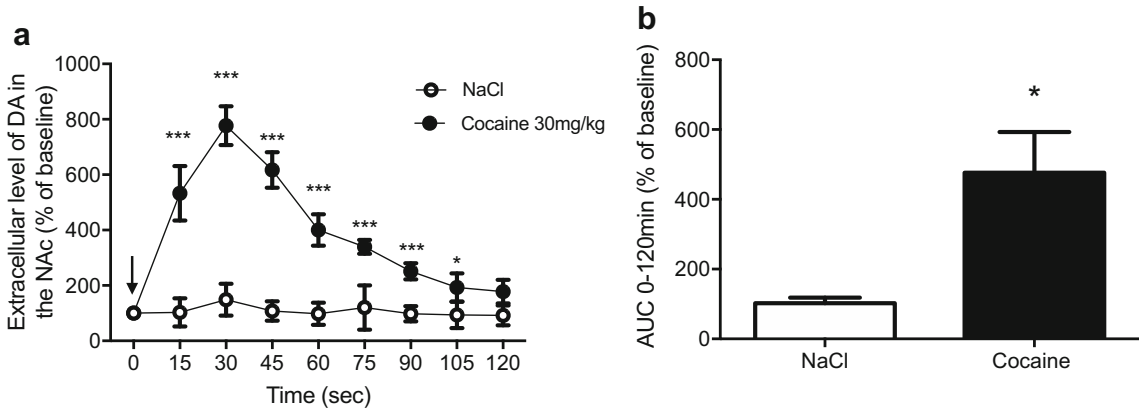

Fig. 6 Effects of an acute injection of cocaine on extracellular DA concentration in the nucleus accumbens. Mean ± SEM of extracellular DA levels expressed as % of baseline in saline (NaCl, s.c., $n = 8$) (white) or cocaine-injected mice (30 mg/kg, s.c., $n = 8$) (black) (**a**). The arrow indicates the time at which mice were injected. As expected, before saline or cocaine injection, the mean basal levels of [DA]ext were not significantly different between groups (2.6 ± 0.4 vs. 3.1 ± 0.8 pg/15 μL respectively, $p > 0.05$). *$p < 0.05$, ***$p < 0.001$: significantly different from saline-injected mice at the corresponding time. Mean ± SEM of area under the curve values following the administration of saline (NaCl) or cocaine in percentage of baseline (**b**). *$p < 0.05$: significantly different from saline-injected mice

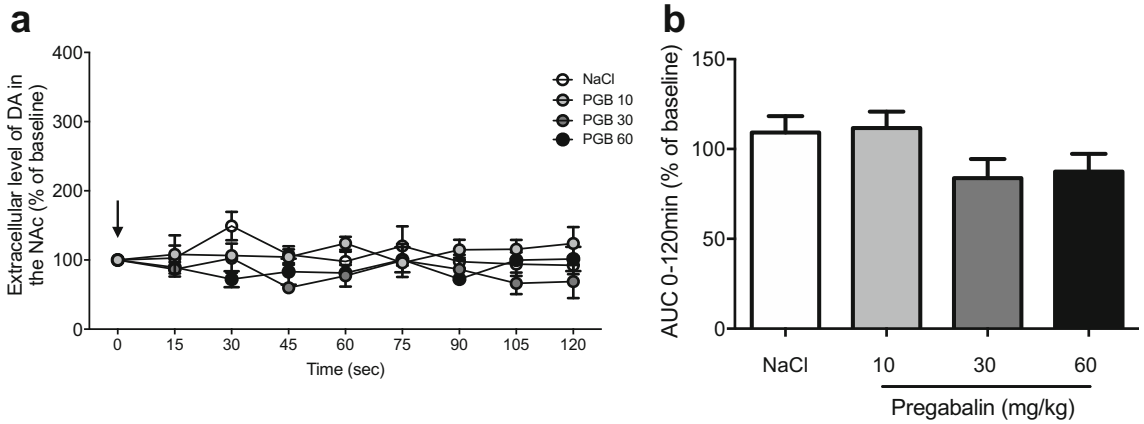

Fig. 7 Effects of an acute injection of pregabalin on extracellular DA concentration in the nucleus accumbens. Mean ± SEM of extracellular DA levels expressed as % of baseline in saline (NaCl, s.c., $n = 8$) (white ○), PGB 10 ($n = 4$, light gray ○), pregabalin PGB 30 ($n = 8$, dark gray ○), and PGB 60 mg/kg ($n = 8$, ●) (**a**). The arrow indicates the time at which mice were injected. As expected, the mean basal levels of [DA]ext was not significantly different between groups (2.6 ± 0.4, 2.9 ± 0.4, 2.0 ± 0.5 et 2.6 ± 0.4 pg/15 μL, respectively). Mean ± SEM of area under the curve values following the administration of saline (NaCl) or PGB in percentage of baseline (**b**)

was similar between PGB- and NaCl-injected animals ($p = 0.65$; Fig. 8b). Similarly, burst frequency and the number of spikes per burst did not differ between groups ($p = 0.3$ and $p = 0.3$, respectively; Fig. 8c–d).

In microdialysis experiments, basal [DA]ext in the NAc in response to repeated administration of PGB did not reveal significant difference relative to saline (2.6 ± 0.4 vs. 2.6 ± 0.5 pg/15 uL, $p = 0.9$). Two-way ANOVA (treatment × time) on the [DA]ext in the NAc failed to unveil significant main effects of treatment factor ($F_{(1,13)} = 0.0812$, $p = 0.78$), time factor ($F_{(8,104)} = 1.808$, $p = 0.08$), and their interaction ($F_{(8,104)} = 0.5612$, $p = 0.8$). Similarly, statistical analysis on AUC (t0–t120 min) values did not show any difference between saline and PGB 60 ($p = 0.63$; Fig. 8e–f).

Discussion

Although many authors contributed to the understanding of the mechanisms by which psychoactive substances produce their potent rewarding effects, there is still some uncertainty concerning some of these pharmacological agents such as PGB. Convergent evidence implicates the meso-accumbens DA neuronal system as a key brain circuit involved in abuse potential (Volkow and Morales 2015). For example, in the operant drug self-administration paradigm, which shows high predictive validity assessing the rewarding properties in rodents and nonhuman primate, DA receptor agonists are known to be self-administered systemically as well as locally into the NAc (Yokel and Wise 1978; Woolverton et al. 1984; Carlezon et al. 1995). On the contrary, DA receptor antagonists given in low doses decrease the motivation to carry out high work requirements to obtain an infusion of cocaine (Hubner and Moreton 1991; Richardson et al. 1993). In agreement with the latter observations, lesions or inactivation of the mesolimbic DA system in the VTA (Roberts and Koob 1982; Shoaib et al. 1998) or the NAc (Roberts et al. 1977; Pettit et al. 1984; Shoaib et al. 1998) attenuate cocaine self-administration in rats. More recently, the emergence of new technologies such as opto- and chemogenetic tools has confirmed the key role of the meso-accumbens DA circuit in the rewarding effects of cocaine (Larson et al. 2015). Here, we used the conditioned place preference (CPP) test to compare the abuse potential of cocaine and PGB. The CPP test is a form of conditioning used to measure the motivational effects of drugs by quantifying the amount of time an animal spends in a compartment that has been associated with this stimulus. In particular, such paradigm has been successfully used to unveil the rewarding properties of dopaminergic compounds (Campbell et al. 2000; Childs and de Wit 2009). Our results indicate a marked place preference for the cocaine-paired side. The highest dose of PGB (i.e., 60 mg/kg) produced similar effects albeit less pronounced (66% vs. 55%; respectively). Although the weak effect detected with PGB60 argues against a robust abuse liability in mice, it is noteworthy that a large proportion of mice displayed an increased percentage of time spent in the drug-paired compartment after conditioning compared to baseline as observed with cocaine (Supp. Fig. 1). Interestingly, when assessing locomotor activity in the CPP in the absence of drug (pre-conditioning vs. post-conditioning sessions), we observed that the PGB60 group, similarly to the cocaine group, showed an increase in locomotion. This conditioned locomotion phenomenon has been associated with cue-induced craving (Hotsenpiller and Wolf 2002) further suggesting that PGB could display rewarding properties at the dose of 60 mg/kg. However, in contrast to cocaine, this drug-free conditioned effect is not accompanied by an acute hyper-locomotor activity in response to PGB administration in the open field. It is also plausible that the effects of PGB would have been more pronounced at higher doses or in

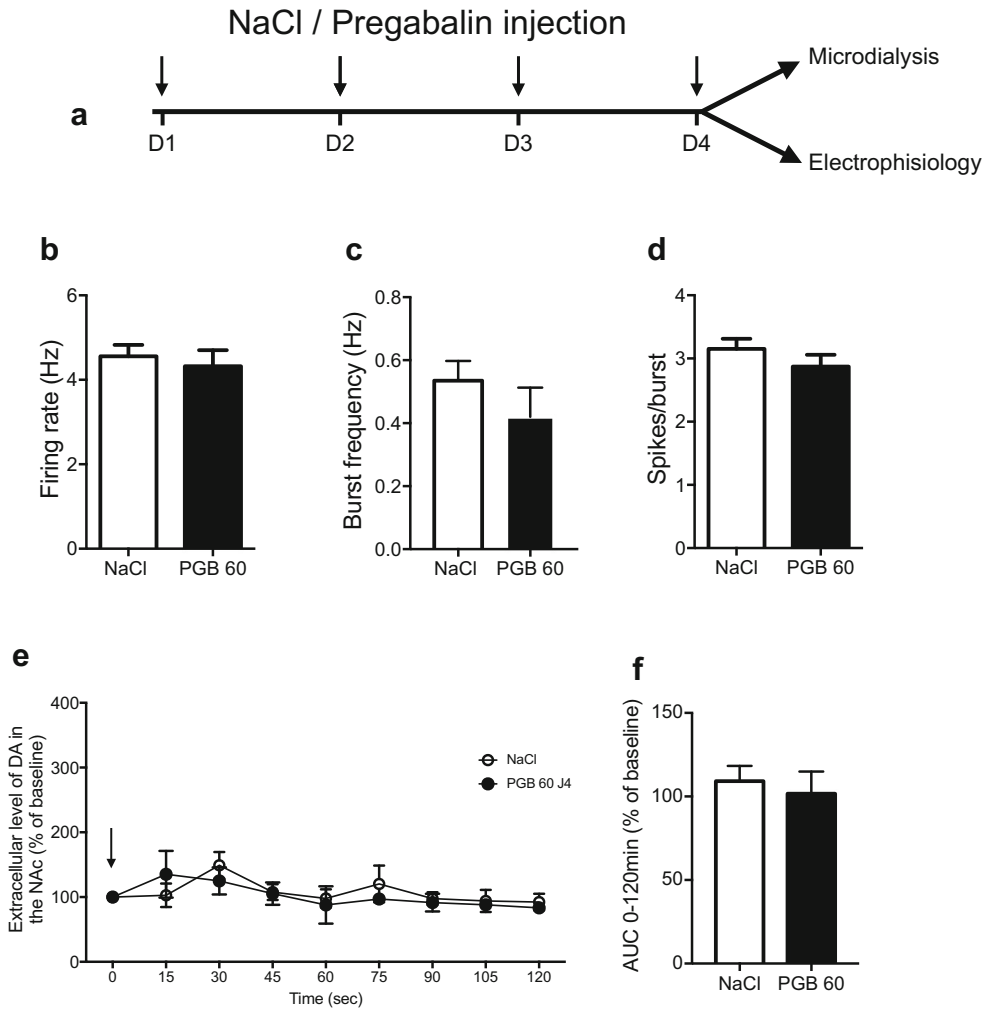

Fig. 8 Effect of repeated administration of pregabalin on dopaminergic activity system. Experimental protocol. Mice received a daily injection of PGB (60 mg/kg; s.c.) or NaCl (0.9%) for 4 days (**a**). Electrophysiological experiments were conducted on day 4 (D4) 30 min after the last injection of PGB. Microdialysis experiments were also conducted on D4 and the last injection of PGB was realized during the samples collection. Mean ± SEM of firing rate (Hz) (**b**), burst frequency (Hz) (**c**), and number of spikes per burst (**d**) after repeated administration of saline ($n = 51$, white) or pregabalin 60 mg/kg, s.c. ($n = 17$, black). Mean ± SEM of extracellular DA levels expressed as % of baseline in saline (NaCl, s.c., $n = 8$) (white) or PGB-injected mice (60 mg/kg, s.c., $n = 7$) (**e**). The arrow indicates the time at which mice were injected. As expected, before saline or cocaine injection, the mean basal levels of [DA]ext were not significantly different between groups (2.6 ± 0.4 vs. 2.6 ± 0.5 pg/15 μL respectively). Mean ± SEM of area under the curve values following the administration of saline (NaCl) or pregabalin (PGB) in percentage of baseline (**f**)

experimental conditions displaying more salient and discriminant cues between CPP compartments.

To our knowledge, there are only few studies evaluating the rewarding properties of PGB and the results are mixed. In rats, an initial study reported that an oral administration of PGB (3–30 mg/kg) had no effect in the CPP test (Andrews et al. 2001). However, these results have been challenged and a preference for the compartment paired with PGB was unveiled in response to its intraperitoneal administration at the doses of 3 and 10 mg/kg (Rutten et al. 2011). Our results obtained in mice with a higher dose of PGB agree with the latter study. The weak percentage of preference for the compartment that has been associated with PGB can be however puzzling and it is possible that the rewarding properties of PGB might be increased under specific experimental conditions. In keeping with this hypothesis, the rewarding effects of chronically administered PGB (3 mg/kg) were unveiled in a mouse model of neuropathic pain based on a partial sciatic nerve ligation (Bura et al. 2018). In a similar model, intraperitoneal injections of PGB (3 mg/kg) also induced place preference during the early stage of neuropathic pain (Asaoka et al. 2018). Finally, rewarding properties have been unveiled after carrageenan-induced mechanical hypersensitivity confirming the abuse potential of PGB (Rutten et al. 2011) although the possible antinociceptive effect of PGB in these models could also account for a place preference. It has also been speculated that PGB could modify the rewarding effects of other drugs of abuse. Different works described reduction in cocaine or alcohol self-administration in rats and humans treated with PGB (Stopponi

et al. 2012; de Guglielmo et al. 2013). In a recent study aimed at deciphering the interactions of PGB and morphine, it was demonstrated that pre-treatment with PGB suppressed morphine self-administration (Hasanein and Shakeri 2014). Conversely, PGB was shown to contribute to oxycodone overdose deaths by reversing oxycodone-induced tolerance and significantly increasing respiratory depression in mice (Hill et al. 2018). Together, these data show that PGB has the capacity of counteracting the rewarding effects of opioids, but it also has a potentiating effect when given to mice with existing low opioid levels (Vashchinkina et al. 2018). It is noteworthy that potential differences in the effects of PGB might exist between mice and humans. However, evidence demonstrates that the pharmacological targets of PGB are the same between both species and that its action in animals and humans is confined to neuronal synapses (Taylor et al. 2007; Li et al. 2011).

Assuming the meso-accumbens DA neuronal circuit as an important component of rewarding properties, we then determined the effects of cocaine and PGB on this system using complementary electrophysiological and neurochemical approaches. We first investigated the effects of cocaine on the firing rate of VTA DA neurons. As previously shown (for review see White 1990), cocaine reduced by ~20% the discharge of VTA DA neurons. Such effect results from the inhibition of DA reuptake. Indeed, this process is responsible for an accumulation of DA around dopaminergic neurons cell bodies, which in turn, activates an inhibitory feedback exerted by the somatodendritic D2 autoreceptors (White 1990). The partial decrease in VTA DA neuronal activity observed here in response to cocaine can appear somewhat surprising notably owing to the high affinity of this drug for the dopamine transporter (DAT) (Uhl et al. 2002). However, there is considerable amount of data demonstrating that the apparent lack of complete inhibition of DA neurons is a feature of this cell population. Indeed, the systemic administration or local application of selective D2 receptor agonists in the VTA produces a partial decrease in the firing rate of DA neurons (Aghajanian and Bunney 1977; White and Wang 1984). It is also noteworthy that non-selective DA re-uptake inhibitors such as GBR12909, SEP225289, or DOV216303 (Einhorn et al. 1988; Guiard et al. 2011) produce only weak inhibition of VTA DA neurons. Cocaine is a non-selective DAT inhibitor which also inhibits the re-uptake transporters of serotonin (SERT) and norepinephrine (NET). The excitatory serotonergic and noradrenergic innervation of the VTA (Adell and Artigas 2004; Esposito 2006) notably mediated by 5-HT2C (Pessia et al. 1994; Di Giovanni et al. 2000; Di Matteo et al. 2000) and alpha1-adrenergic receptors (Grenhoff et al. 1995; Steffensen et al. 1998) have been proposed as key processes to limit the inhibitory effect of acutely administered cocaine on VTA DA neuronal activity. Using intracerebral microdialysis in awake freely moving mice, we showed that cocaine elicits a robust increase (800%) in extracellular DA levels in the NAc. Different microdialysis studies in the behaving rodents had previously yielded similar results demonstrating that the acute systemic administration of cocaine (Hernandez and Hoebel 1988; Bradberry and Roth 1989; Kalivas and Duffy 1990; Kuczenski et al. 1991; Pettit and Justice 1991) but also other DAT inhibitors such as GBR12909 (Tsukada et al. 2000) or amphetamine (Di Ciano et al. 1995), increases DA levels in the NAc. Taken together, our results strongly suggest that the rewarding properties of cocaine resulted from an increased extracellular DA levels in the NAc in response to the inactivation of the DAT.

As regards PGB, our data revealed the existence of a subpopulation of VTA DA neurons that are inhibited by ~50% at the dose of 60 mg/kg. This electrophysiological response could suggest that PGB operates through the inactivation of the DAT as cocaine does. However, the fact that PGB did not increase extracellular DA levels in the NAc is not in favor of this hypothesis. Alternative mechanisms have to be foreseen. In particular, considering the excitatory serotonergic and noradrenergic innervation of the VTA, the possible interactions of PGB with both transmissions would deserve to be investigated in future research. Moreover, given the affinity and blocking activity of PGB on the $\alpha_2\delta_1$ subunit of Ca^{2+} voltage-gated channels (Ben-Menachem 2004), it is possible that the observed reduction in firing rate resulted from a decreased inward Ca^{2+} current in the VTA DA neurons thereby limiting cell depolarization but this hypothesis cannot explain the rewarding properties of PGB observed in the CPP test. Interestingly, our study also highlighted a subpopulation of VTA DA neurons (~30%) that were excited by PGB. Therefore, the rewarding properties of PGB could specifically rely on and be limited to these cells. One would expect that PGB inhibited Ca^{2+} voltage-gated channels expressed on GABAergic neurons thereby producing a disinhibitory action upon this subpopulation of VTA DA neurons. Another avenue that could be explored is the putative action of PGB on glia. Recent data demonstrate that microglia disrupts the mesolimbic system (Taylor et al. 2015). Assuming that PGB would modulate the activity on these non-neuronal cells as previously observed (Daneshdoust et al. 2017), it would be tempting to examine this indirect relationship between PGB and VTA DA neurons. In a last series of experiments, we showed that repeated administration of PGB for 4 consecutive days at the dose of 60 mg/kg (chosen to mimic the CPP test conditions) did not influence the DA system. Previously, PGB (3 mg/kg) was shown to induce self-administration in the operant test after a 10-day period treatment (Bura et al. 2018) suggesting that a longer exposure to PGB may have been necessary to obtain a significant impact on DA levels in the NAc. However, the absence of elevated concentrations of DA in this brain region was not detrimental to obtain a behavioral response in the CPP in the present study.

To further improve the understanding of the abuse/addiction potential of PGB, a complementary transversal

approach should be implemented, in which animal models would be used to mimic different pathophysiological states found associated to PGB abuse in humans (Asomaning et al. 2016; Driot et al. 2016b; Schjerning et al. 2016; Evoy et al. 2017). Exposure to methadone, which constitutes an abuse/addiction marker, is an important factor of susceptibility identified in observational studies. So, to complement the results obtained in healthy mice, the mechanisms underlying PGB's abuse/addiction potential should be investigated in animal models of dependence using experiments as in the present study. Indeed, the interaction between use of opioids and PGB is undeniable but complex. The analysis of postmortem toxicological data considered the increasing trend of PGB abuse and concluded on the mortality risk when PGB was combined with opioids, which were found concomitant in 91.4% of cases (Hakkinen et al. 2014).

Conclusion

A better understanding of the mechanisms involved in PGB's abuse/addiction would help clinicians detect these disorders in their patients as well as manage them. A recent observational study found that 12.1% of opioid-dependent patients followed in an addictology unit were also using PGB although they presented no medical indication to use it (Grosshans et al. 2013). Moreover, causes of sudden suspect deaths in individuals aged from 15 to 34 years old were investigated in Finland (Launiainen et al. 2011). Overall, 10% of these deaths could be associated to PGB intake, 62% of which in a context of abuse/addiction. Surprisingly, this value was lower as opioids were concerned (fentanyl 56%, tramadol 55%, morphine 43%). In England, in 2018, the study of non-medical use of prescription drugs identified PGB as one of the five psychoactive prescription drugs associated with fatal poisoning among 14 studied (OR 2.60, 95% confidence interval (CI) 1.61–4.20), after fentanyl (OR 4.25, CI 1.76–10.23) and before oxycodone (OR 2.51, CI 1.51–4.19), tramadol (OR 2.23, CI, 1.49–3.35), and alprazolam (OR 1.76, CI1.18–2.60) (Haukka et al. 2018). It is thus essential to widespread the knowledge on this abuse/addiction potential among health professionals but also in the general public, to raise awareness on the risks for PGB abusers as well as for patients being prescribed PGB, which are currently underestimated or even unknown. One consequence of this lack of consciousness is the underreporting of abuse cases to national or international pharmacovigilance systems resulting in the inability of these systems to identify PGB's abuse potential (Bossard et al. 2016).

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Compliance with ethical standards

All experiments were carried out in accordance with the European guidelines for the care of laboratory animals (European Communities Council Directive 86/609/ECC) and approved by the local Ethics Committee and the French Ministry of Education and Research (APAFIS#6659-2016090512114815 v3).

Conflict of interest The authors declare that they have no conflict of interest.

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