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Adrenergic Receptor Agonists' Modulation of Dopaminergic and Non-dopaminergic Neurons in the Ventral Tegmental Area

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Abstract—The ventral tegmental area (VTA) neuronal population consists of dopaminergic (DAergic) and non-DAergic neurons (mainly GABAergic), the activity of which is intertwined with VTA behavioral functions. Both DAergic and GABAergic neurons in the VTA have been shown to express adrenergic receptors (ARs) and respond to AR stimulation. The aim of the present study was to demonstrate the effects of selective AR agonists on DAergic and non-DAergic neuronal activity in the central and lateral parts of the VTA using *in vivo* electrophysiological recording combined with microiontophoretic drug application in anaesthetized rats. Administration of phenylephrine, a selective α_1-AR agonist, while having an inhibitory effect on putative DAergic neurons (11% decrease in firing rate), induced a clear excitatory effect (59% increase in firing rate) on putative non-DAergic neurons. In contrast, application of clonidine, a selective α_2-AR agonist, or isoprenaline, a selective β-adrenergic receptor agonist, did not change the firing rate of either DAergic or non-DAergic neurons but influenced the firing pattern of non-DAergic cells only. Our results suggest that noradrenaline modulates activity of VTA neurons *in vivo* primarily via α_1, but also via β- and α_2-AR to a lesser extent. Furthermore, we show that α_1-AR activation has contrasting effects on putative DAergic and non-DAergic neurons. We hypothesize that the phenylephrine-induced inhibition of putative DAergic neurons results from activation of GABAergic terminals present at the site of drug application. Such a mechanism is further supported by the observed α_1-AR-induced excitation of putative GABAergic VTA neurons.

Key words: ventral tegmental area, dopamine, noradrenaline, burst activity, alpha$_1$-adrenergic receptor.

INTRODUCTION

The ventral tegmental area (VTA) is a centrolateral part of the A10, the largest group comprising dopaminergic (DA) neurons in mammalian brain. The role of the VTA neuronal activity has been extensively studied in recent decades, particularly focusing on the activity of DAergic neurons, which led to the formation of the reward prediction error (RPE) model of reinforcement learning (Schultz, 1998, 2016). However, the VTA neuronal population is heterogeneous as it consists of DAergic (~67%), GABAergic (~30%) and glutamatergic (~2–3%) neurons (Nair-Roberts et al., 2008; Ungless and Grace, 2012). Thus, the VTA non-DAergic neurons are predominantly GABAergic, as opposed to the medial parts of A10, which, apart from DAergic neurons, contain predominantly glutamatergic cells (Yamaguchi et al., 2015).

DAergic, as well as GABAergic and glutamatergic neurons, send projections to target forebrain structures, such as the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), and support diverse functional roles (Carr and Sesack, 2000a,b; Omelchenko and Sesack, 2009; Taylor et al., 2014). Apart from these distant projections of VTA GABAergic neurons, these cells form local synapses within the VTA (Omelchenko and Sesack, 2009), and their activation potently inhibits firing of DAergic neurons (van Zessen et al., 2012). Indeed, regulation of VTA GABAergic neuronal activity has meaningful implications for behavior. Recent studies have demonstrated that RPE encoding correlates with both

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Abbreviations: AR, adrenergic receptor; CLON, clonidine; DA, dopamine; EPSC, excitatory postsynaptic current; GABA, γ-aminobutyric acid; IP, isoprenaline; IPSC, inhibitory postsynaptic current; LC, locus coeruleus; mPFC, medial prefrontal cortex; NA, noradrenaline; NAc, nucleus accumbens; PE, phenylephrine; RPE, reward prediction error; sEPSC, spontaneous excitatory postsynaptic current; sIPSC, spontaneous inhibitory postsynaptic current; VTA, ventral tegmental area.

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DA and GABAergic neuronal activity, leading to the hypothesis that conditional stimuli and outcome expectations are encoded by DA and GABAergic neurons, respectively (Cohen et al., 2012; Eshel et al., 2016). Similarly, many environmental stimuli regulate DA signaling indirectly via modulation of the activity of VTA GABAergic neurons. For example, administration of a μ opioid receptor agonist (e.g., morphine) leads to VTA DAergic neuron disinhibition, supporting opioid-induced reinforcement learning (Johnson and North, 1992a,b). Other drugs of abuse such as ethanol, nicotine, benzodiazepines and tetrahydrocannabinol also increase DA release via regulation of the VTA GABAergic neurons (Mansvelder et al., 2002; Mansvelder and McGehee, 2002; Szabo et al., 2002; Stobbs et al., 2004; Tolu et al., 2013; Marti-Prats et al., 2015). Exposure to stressful stimuli also increases activity of the VTA GABAergic neurons; however, brief excitation of DAergic neurons has also been reported (Anstrom and Woodward, 2005; Anstrom et al., 2009; Brischoux et al., 2009; Gurraci and Kapp, 1999). The above observations indicate that different brain structures send functional inputs synapsing with distinct VTA neuronal populations.

The noradrenergic (NA) system is well positioned to control the activity of distinct VTA neuronal populations both at the level of midbrain cell bodies and axon terminals innervating the VTA (Geisler and Zahm, 2005; Masana et al., 2011). In addition, studies using both anterograde and retrograde tracing techniques have proven an anatomical connection between the NAergic brain nuclei. Both the area A1 and A2 cell groups, situated within the caudal part of the nucleus of the solitary tract and the locus coeruleus (LC), the main source of NA in the central nervous system, have been shown to send projections to the VTA (Mejias-Aponte et al., 2009; Rinaman, 2010, 2011; Jones and Moore, 1977; Phillipson, 1979; Simon et al., 1979; Geisler and Zahm, 2005 for detailed neuroanatomy of the noradrenergic system see Robertson et al., 2013). Interestingly, the low level of monosynaptic LC NAergic inputs to DAergic neurons in the VTA (Watabe-Uchida et al., 2012) suggests that NAergic signaling might primarily affect the activity of non-DAergic neurons. On the other hand, the infrequent direct synaptic contacts between NAergic fibers and DAergic neurons might not be surprising since NA is mainly released by means of volume transmission (Pickel et al., 1996; Mejias-Aponte, 2016). Indeed, terminals expressing the NA transporter form predominantly nonsynaptic contacts with TH-immunoreactive dendrites in the VTA, which suggests that NA released from varicosities modulates DAergic neurons in the VTA mainly in a paracrine manner (Liprando et al., 2004).

The NA is a potent modulator of neuronal activity by acting through G-coupled receptors such as α_1-, α_2- and β-adrenergic receptors (ARs). Activation of ARs have been shown to modulate VTA neuronal activity (Aghajanian and Bunney, 1977; White and Wang, 1984; Grenhoff et al., 1995; Paladini and Williams, 2004; Arencibia-Albite et al., 2007; Guiard et al., 2008a; Inyushin et al., 2010; Jimenez-Rivera et al., 2012; Velasquez-Martinez et al., 2012, 2015; Williams et al., 2014; Goertz et al., 2015). Importantly, recent studies have suggested that intra-VTA NAergic signaling might indeed effectively regulate VTA-dependent behavioral functions (Goertz et al., 2015; Solecki et al., 2017a,b).

Mechanistically, NAergic signaling in the VTA modulates neuronal activity presumably via α_1-AR. The majority of α_1-ARs are found in the VTA are located presynaptically on unmyelinated axons and axon terminals of glutamatergic and GABAergic neurons. However, α_1-ARs are also found on neuronal dendrites and glial cells in the VTA (Rommelfanger et al., 2009; Mitrano et al., 2012). In addition, α_2- and β-AR radioligand binding in the VTA has also been reported, suggesting receptor-specific VTA regulation (Rainbow et al., 1984; Rosin et al., 1993; Lee et al., 1998).

Increasing literature demonstrates a variety of effects of ARs on DAergic neuronal activity. Systemic administration of prazosin, an α_1-AR antagonist, decreases bursting of DAergic neurons (Grenhoff and Svensson, 1993). Additionally, stimulation of the LC leads to a short increase in DAergic neuronal activity followed by an activity pause (Grenhoff et al., 1993), whereas systemic administration of prazosin attenuates this effect. In contrast, lesion of the LC leads to a robust increase in the firing rate and bursting of VTA DAergic neurons (Guiard et al., 2008b). In addition, systemic clonidine administration (α_2-AR agonist) leads to regularization of DAergic cell firing (Grenhoff and Svensson, 1988, 1989), whereas an α_2-AR antagonist leads to an increase in firing rate and bursting (Grenhoff and Svensson, 1988, 1989, 1993). These studies strongly demonstrated that NAergic signaling regulates VTA DAergic activity; however, these effects were mediated via whole-brain network alterations, and the exact brain locus was not identified.

More detailed studies have demonstrated that presynaptic α_1-AR activation in the VTA enhances glutamate release (Velasquez-Martinez et al., 2012) and decreases GABA release (Velasquez-Martinez et al., 2015), potentially leading to increased VTA neuronal activity. Similarly, postsynaptic α_1-AR activation enhances the activity of both DAergic (Grenhoff et al., 1995; Williams et al., 2014; Goertz et al., 2015) and non-DAergic neurons (Grenhoff et al., 1995). Accordingly, *in vivo* pressure application of an α_1-AR agonist resulted in enhancement of DAergic neuronal activity (Goertz et al., 2015). In contrast, other studies conducted *in vivo* show no effects of an α_1-AR antagonist (White and Wang, 1984) or report that α_1-AR activation leads to inhibition of DAergic neurons (Paladini and Williams, 2004) and elevation of the sIPSP frequency observed in DAergic cells (Grenhoff et al., 1995).

The above studies demonstrate opposing effects of α_1-AR activation on the activity of VTA DAergic neurons. Despite the established NAergic innervation, as well as the expression and location of ARs in the VTA, the receptor mechanisms that regulate VTA neuronal activity are poorly understood. Moreover, the majority of the VTA α_1-AR effects have been shown using *in vitro* electrophysiological recordings, and the field lacks

knowledge of how local activation of different ARs affects the activity of both DAergic and non-DAergic VTA neurons *in vivo*. Thus, in our study, we aimed to examine the effects of local iontophoretic application of α_1-, α_2- or β-AR selective agonists on the activity of putative DAergic and putative non-DAergic neurons in the central and lateral parts of the rat VTA.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (270–430 g; $n = 17$) were acquired from the Institute of Pharmacology PAS (Krakow, Poland) breeding facility. Animals were housed five per cage in a temperature and humidity-controlled room (20–22 °C, 40–50% humidity) on a 12-h light/dark cycle (lights on at 7 am) with *ad libitum* access to food and water. Before any surgical procedures, rats were allowed to acclimate to the facility for two weeks. All experiments were performed during the light phase of the cycle. All experimental procedures were conducted according to the EU Guide for the Care and Use of Laboratory Animals and were approved by the Committee on the Ethics of Animal Experiments at Jagiellonian University.

Surgical procedure

All surgical procedures were performed under deep anesthesia induced by intraperitoneal injection of urethane (1.5 g/kg, Sigma–Aldrich, Germany) diluted in 0.9% sodium chloride. The rat body temperature was monitored by an automatic heating pad (temperature controller TCP-02; WMT, Poland) via a rectal thermometer and held at 37 °C. Animals were carefully mounted on the standard ear (EB-918, 18 ° tip) and incisor bars (RA-200) in a stereotaxic frame (SF-1450AP, ASI Instruments Inc.; Warren, MI, USA). A sagittal incision on the top of the head was performed, and the skin and soft tissue covering the bones were retracted to expose the sutures. After ensuring that bregma and lambda were evenly positioned in the dorso-ventral axis, craniotomies were performed to allow for implantation of electrodes used for electrocorticographic and single-cell recordings. All exposed brain surfaces were covered with mineral oil to prevent tissue drying.

Recording and iontophoretic drug application

Extracellular recordings were conducted using 3- and 4-barrel iontophoretic micropipettes prepared from borosilicate glass capillaries (o.d.: 1.5 mm, i.d.: 0.86 mm, with internal filament for better electrode filling; Sutter Instrument USA). Capillaries were tightened with heat-shrinkable tube and then twisted and pulled on a vertical puller (model PE-21, Narishinge International Instruments, Japan). The recording barrel was filled with 2% Chicago Sky Blue in 2 M NaCl (5–10 MΩ impedance at 1 kHz, measured in situ). The current balancing barrel was filled with 2 M NaCl solution to compensate for ion efflux/influx. Drug application barrel(s) were filled with adrenergic agonists (Sigma–Aldrich, Germany): 100 mM phenylephrine (α_1-AR agonist; PE), 100 mM isoprenaline (β-AR agonist; IP) or 100 mM clonidine (α_2-AR agonist; CLON) depending on the experiment. Drugs were diluted in distilled water, and the pH was adjusted using 1 M HCl to a value of 4.2–4.5 for proper ionization of compounds. Agonist solutions were then portioned and stored at −20 °C. During iontophoretic drug application, a positive current in a range of 8–30 nA was used for ejection, and a negative current in a range of 10–15 nA was used to retain the substance in the pipette. Drug application/retention currents were controlled by a 2-channel iontophoresis amplifier with automatic balance (MVCS-02C, NPI electronic GmbH, Germany). The drug application times ranged from 40 to 100 s (depending on the level of current injection-induced noise). The timing and amplitude of currents were digitally operated and monitored using a 1401 mkII interface (Cambridge Electronic Design; Cambridge, UK). The efficacy of iontophoretic drug application was confirmed by control experiments performed on red nucleus, a neuronal population with defined noradrenergic inputs, marked AR expression, and well-established sensitivity to AR agonists (Swanson and Hartman, 1975; Mejias-Aponte et al., 2009; Wanaka et al., 1989; Ciranna et al., 1996; Domyancic and Morilak, 1997). (Fig. 1). Such control applications were performed during most of the experiments as the red nucleus is positioned just above the VTA. Electrocorticographic recordings (ECoGs) were conducted using a silver-ball electrode (0.1–0.2 MΩ measured at 1 kHz in saline; 0.7 mm in diameter) located epidurally over the right hemisphere at the border of the primary motor and somatosensory cortices. The ECoG was performed to avoid drug application during changes of sleep phases, as VTA neuronal activity has been shown to differ with alternating brain states of urethane anaesthetized rats (Walczak and Blasiak, 2017). The tip of the recording pipette was positioned in the VTA using the following stereotaxic coordinates: 5.0 to 6.0 caudally from bregma, 0.6–1.0 laterally from the middle of the sagittal sinus, and 7.8–9.0 ventrally from the skull surface at the bregma point (Paxinos and Watson, 2007). Signals were amplified and filtered (single-unit activity: 10,000×, 300–5000 Hz; ECoG: 1000x, 1–500 Hz) with an A-M Systems micro-electrode amplifier (model 1800; Carlsborg, WA, USA). Analog signals were digitized with a micro 1401 mkII interface equipped with Spike2 software for storage and further analysis (Cambridge Electronic Design; Cambridge, UK). After the end of the recordings, a negative current of 15 μA was passed for 15 min through the recording pipette to deposit dye marking the recording site (Stimulus Isolator; WMT, Poland).

Identification of dopaminergic neurons

Neurons were classified as dopamine-like when they met the previously established electrophysiological criteria (Grace and Bunney, 1980, 1983; Ungless and Grace, 2012): a broad (> 1.1 ms) triphasic action potential (length measured from action potential initiation to minimum of the trough) and a firing rate below 10 Hz. Neurons

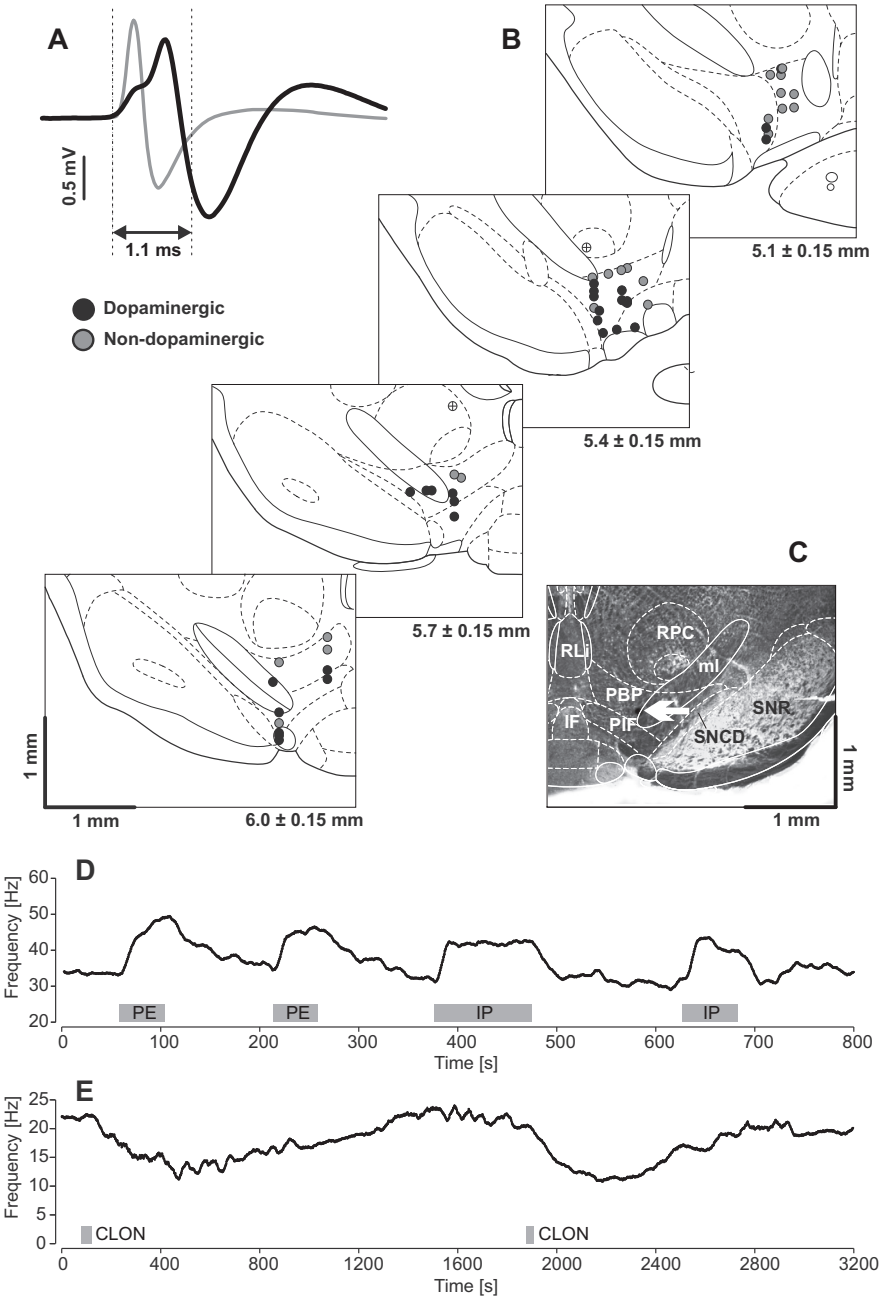

Fig. 1. Histological verification of *in vivo* recordings and control adrenergic receptor agonist applications. (A) Typical action potential shape of a putative DAergic (black) and non-DAergic (gray) VTA neuron. (B) Localization of neurons that were recorded and used for statistical analysis. Black dots depict putative DAergic neurons and gray dots represent putative non-DAergic neurons, whereas crossed dots represent control neurons located in the red nucleus. Cells are depicted on one hemisphere; however, recordings were performed in both VTAs. The number near each coronal section represents the distance in mm from Bregma in the antero-posterior axis. (C) Example of the histological slice overlain with corresponding section from the Stereotaxic Atlas, which allowed for precise neuronal mapping. Chicago Sky Blue dye deposition is marked with an arrow. (D) Iontophoretic application of phenylephrine (PE) or isoprenaline (IP) on neuron located in the red nucleus. (E) Iontophoretic application of clonidine (CLON) on neuron located in the red nucleus. Gray bars on D and E indicate the time of drug application.

that did not follow at least one of these criteria were classified as non-dopaminergic. Final classification of neurons as putative DAergic was performed after histological confirmation of recording sites within the VTA.

Histological verification

At the end of the experiments, animals were sacrificed by decapitation. The brains were extracted, held in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for at least 48 h, and then sliced at 50 μm in the coronal plane using a vibratome (Leica VT1000S, Heidelberg, Germany). Slices containing dye depositions were photographed and adjusted to the corresponding section of the stereotaxic atlas of the rat brain (Paxinos and Watson, 2007) using CorelDRAW software to verify recording placements. The positions of all recorded DAergic and non-DAergic neurons were located on the coronal diagrams encompassing the dopaminergic, midbrain subregions.

Data analysis

For each putative DAergic and non-DAergic neuron, the spontaneous basal activity was recorded for at least 100 s before drug application. For further analysis, files containing neuronal activity were manually inspected using Spike2 software to ensure that no action potentials were skipped or false action potentials were added to the analyzed signal. Two 40-s-long periods were used for analysis, one referring to the basal activity (just before the drug application) and one during adrenergic agonist application (last 40 s of application). Calculation of the action potential duration, spontaneous firing rate, analysis of interspike intervals, detection and analysis of bursts of action potentials and the extraburst firing rate were performed off-line, using custom-made Spike2 scripts (Cambridge Electronic Design; Cambridge, UK). In case of putative DAergic neurons train of action potentials was classified as a burst when it met the previously established criteria (Grace and Bunney, 1984): time between the initial action potential of the burst and the following one was shorter than or equal to 80 ms, and an interspike interval greater than 160 ms terminated the burst. In case of putative non-DAergic neurons bursts were detected based on spike density function (SDF; gaussian kernel, width 100 ms; MLIB toolbox for MATLAB, MathWorks, Inc., Massachusetts, USA; Szucs, 1998) crossing

the threshold of two standard deviations above mean value. Parameters of detected bursts were determined by custom-made MATLAB script. Analysis of the burst parameters was only performed for cells that generated at least one burst within the subjected period of time. Student's t-test for paired (when each baseline data record had its corresponding agonist application data record) or unpaired (when some of data records were not present either during baseline or after agonist application) data was used in the case of normally distributed samples (Kolmogorov–Smirnov's normality test). In case of data with the not-Gaussian distribution, nonparametric tests were applied (Wilcoxon's matched-pairs or Mann–Whitney's test). Differences were considered significant at $p < 0.05$. All data shown are mean ± SEM. Statistical tests and calculations were performed using Prism 6 software (GraphPad Software Inc., CA, USA).

RESULTS

The activity of 66 neurons was recorded in the VTA of 17 rats. Recording and iontophoretic drug application sites in the VTA were confirmed by histological verification (Fig. 1), and the putative phenotype (DAergic, non-DAergic) of studied neurons was determined based on previously used electrophysiological criteria (Grace and Bunney, 1980, 1983; Ungless and Grace, 2012). Importantly, it has been demonstrated that these criteria fail to work properly in minority of cases (Lou et al., 2008; Brischoux et al., 2009). Therefore, in the present study, the length of the action potentials' shape was measured from its beginning to the peak of the negative through, as it gives better output of proper neuronal classification (> 1.1 ms for DAergic neurons) than measuring the whole action potential (Ungless et al., 2004; Mileykovskiy and Morales, 2010; Moorman and Aston-Jones, 2010) (DA neurons: 1.41 ± 0.23 ms, non-DA neurons: 0.61 ± 0.11 ms). Putative DAergic neurons in the VTA ($n = 36$) displayed a spontaneous firing rate of 4.7 ± 0.3 Hz, whereas the average spontaneous firing rate of putative non-DAergic neurons ($n = 30$) was 7.2 ± 1.4 Hz. The spontaneous activity of putative DAergic and non-DAergic neurons was similar to that described previously in the literature (Marinelli and McCutcheon, 2014).

α_1-Adrenergic receptor agonist effects on VTA neuronal activity

A representative example of a putative DAergic neuron response to iontophoretic phenylephrine application is shown in Fig. 2A. The average current and time of PE application on putative DAergic neurons is shown in Table 1. PE administration induced a significant decrease in both the total firing (baseline: 4.4 ± 0.6 Hz, PE: 3.9 ± 0.6 Hz, $n = 12$, $p < 0.01$; Fig. 2B; Table 2) and extraburst firing rate (baseline: 3.9 ± 0.5 Hz, PE: 3.5 ± 0.6 Hz, $n = 12$, $p < 0.05$; Fig. 2C; Table 2) of putative DAergic neurons. At the same time, PE application did not induce changes in the intraburst firing rate (baseline: 16.8 ± 1.7 Hz, PE: 16.2 ± 1.3 Hz, $n = 12$, n.s., Fig. 2D; Table 2), as well as other bursting parameters of putative DAergic neurons (i.e.,% of spikes fired in bursts, bursting rate, mean spikes per burst, mean intraburst interspike interval, burst length; Table 3). Such results demonstrate that PE application modulates extraburst activity but has no effect on bursting of putative DAergic neurons. This observation is further complemented by an increase in coefficient of variation induced by phenylephrine application (baseline: 0.72 ± 0.08, PE: 0.84 ± 0.10, $n = 12$, $p < 0.01$; Table 2), as lowered extraburst activity with intact intraburst activity results in bigger contribution of bursting activity on overall regularity of action potentials.

In contrast to the α_1-AR agonist effects on putative DAergic neuronal activity, PE iontophoretic application (average current and time of iontophoretic application in Table 1) induced a significant increase in the firing rate of putative non-DAergic neurons in the VTA (baseline: 8.5 ± 2.7 Hz, PE: 13.5 ± 3.1 Hz, $n = 11$, $p < 0.05$; Fig. 3A, B; Table 4). The PE-induced increase in putative non-DAergic neuronal activity ramped up slowly over time (average peak of excitation at 115 s from the start of PE application) and lasted for at least 200 s (Fig. 3E). At the same time, no changes in coefficient of variation were observed (baseline: 0.87 ± 0.14, PE: 0.84 ± 0.12, $n = 11$, n.s.; Table 4). Additionally, PE application changed bursting parameters of non-DAergic neurons. Intraburst firing rate (baseline: 22.4 ± 3.5 Hz, PE: 33.7 ± 3.7 Hz, $n = 11$, $p < 0.01$; Fig. 3C; Table 5) and mean spikes per burst (baseline: 3.6 ± 0.5, PE: 5.6 ± 0.7, $n = 11$, $p < 0.05$; Fig. 3D, Table 5) were elevated, while mean burst interspike was decreased (baseline: 57.5 ± 9.4 ms, PE: 35.5 ± 5.5 ms, $n = 11$, $p < 0.01$; Table 5). Other bursting parameters remained unaltered (i.e., % of spikes fired in bursts, bursting rate, mean burst length) (Table 5).

Non-DAergic VTA neurons form local synapses with DAergic neurons within the VTA and can influence their activity (Omelchenko and Sesack, 2009; van Zessen et al., 2012). Thus, we tested if the peak excitation of putative non-DAergic neurons induced by PE (115 s from beginning of application; Fig. 3C) coincided with the decrease in firing of the putative DAergic neurons. Therefore, an additional time point (115 s) of DAergic neuronal activity was analyzed. In contrast to the above reported results, no inhibition was detected (baseline: 4.5 ± 0.7 Hz, PE: 4.1 ± 0.7 Hz, $n = 11$, n.s.; Table 2), suggesting a transient PE effect. There were also no differences in the intraburst firing rate (baseline: 16.9 ± 1.8, PE: 15.5 ± 1.0; $n = 11$, n.s.; Table 2) or other bursting parameters (Table 3) as well as no changes in extraburst firing (baseline: 4.0 ± 0.6 Hz, PE: 3.7 ± 0.7 Hz, $n = 11$, n.s.; Table 3) as well as no changes in coefficient of variation were observed (baseline: 0.69 ± 0.09, PE: 0.75 ± 0.09, $n = 11$, n.s.; Table 2). In conclusion, local, iontophoretic application of PE in the VTA resulted in excitation of putative non-DAergic neurons and inhibition of putative DAergic neurons.

α_2-Adrenergic receptor agonist effects on VTA neuronal activity

Iontophoretic application of clonidine (average current and time of iontophoretic application in Table 1) had no

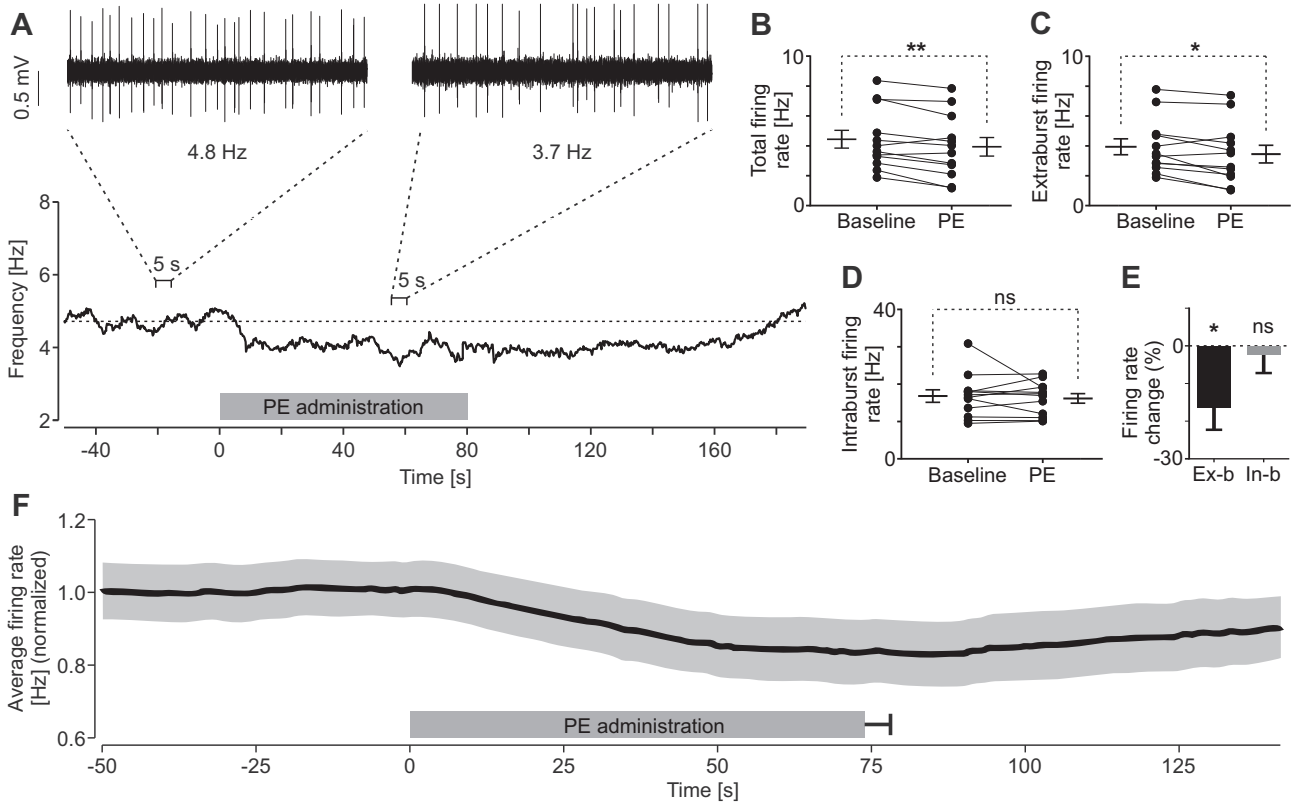

Fig. 2. Phenylephrine (α_1-AR agonist)-induced inhibitory effect on putative DAergic neurons in the VTA. (A) Representative neuronal activity before and after phenylephrine application. (B) Mean ± SEM and individual putative DAergic neurons' total firing rates before (baseline) and after phenylephrine application (PE). (C) Mean ± SEM and individual neurons' extraburst firing rates before (baseline) and after phenylephrine application (PE). (D) Mean ± SEM and individual putative DAergic neurons' intraburst firing rate before (baseline) and after phenylephrine application (PE). (E) Mean ± SEM change (mean treatment to baseline ratios) of the extraburst (ex-b) and intraburst (in-b) firing rate after phenylephrine application. (F) Average activity (normalized to baseline) of all putative DAergic neurons in the VTA after local phenylephrine administration. Beginning of all time axes (0 s) was set to the beginning of drug application. Gray bars on A and F indicate drug application time. Lines or bars with whiskers on B, C, D, E and F indicate mean ± SEM. Black circles in B, C, D and E indicate individual neurons. **For $p < 0.01$, *for $p < 0.05$, ns: non-significant.

Table 1. Average currents and times of iontophoretic drug application on dopaminergic and non-dopaminergic neurons for particular adrenergic agonists

	DA – PE	non-DA – PE	DA – CLON	non-DA – CLON	DA – IP	non-DA – IP
Average time of drug application [s]	73.9 ± 4.2	80.0 ± 5.5	43.5 ± 4.8	53.2 ± 5.4	91.9 ± 11.7	73.0 ± 6.8
Average current of drug application [nA]	18.3 ± 0.7	16.4 ± 1.5	12.0 ± 1.1	11.6 ± 0.5	18.7 ± 0.6	21.3 ± 2.9

effect on the firing rate of both putative DAergic (baseline: 5.1 ± 0.5, CLON: 5.1 ± 0.6, $n = 11$, n.s.; Fig. 4A; Table 2) and non-DAergic neurons (baseline: 8.8 ± 1.7 Hz, CLON: 7.7 ± 1.7 Hz, $n = 6$, n.s., Fig. 4D). In addition, there was no alteration in the bursting activity of putative DAergic neurons as the intraburst firing rate (baseline: 14.4 ± 1.6 Hz, CLON: 13.0 ± 1.0 Hz, $n = 11$, n.s.; Fig. 4C; Table 2) and other bursting parameters (Table 3) did not change after CLON application. Furthermore, no changes in the extraburst firing rate (baseline: 4.9 ± 0.4 Hz, CLON: 4.9 ± 0.5 Hz, $n = 11$, n.s.; Fig. 4B; Table 2) and coefficient of variation were observed (baseline: 0.46 ± 0.07, CLON: 0.45 ± 0.07, $n = 11$, n.s.; Table 2). Small increase in coefficient of variation was detected in case of putative non-DAergic neurons (baseline: 0.41 ± 0.07, CLON: 0.54 ± 0.10, $n = 6$, $p < 0.05$; Table 4), although there was no change in bursting parameters of putative non-DAergic neurons after clonidine application (Table 5). In conclusion, clonidine did not alter the electrophysiological activity of putative DAergic neurons but slightly altered regularity of putative non-DAergic neurons' action potentials leaving the firing rate intact.

β-Adrenergic receptor agonist effects on VTA neuronal activity

Iontophoretic application of isoprenaline (average current and time of iontophoretic application in

Table 2. Effects of selective AR activation on the total, extraburst and intraburst firing rate of dopaminergic neurons

	Drug tested	Baseline	Drug administration	*p*	*t*	*n*
Total firing rate [Hz]	Phenylephrine	4.4 ± 0.6	3.9 ± 0.6	****0.0051**	3.49	12
	Phenylephrine 115 s	4.5 ± 0.7	4.1 ± 0.7	0.071	2.05	11
	Isoprenaline	4.7 ± 0.5	4.6 ± 0.5	0.341	0.99	13
	Clonidine	5.1 ± 0.5	5.1 ± 0.6	0.911	0.12	11
Extraburst firing rate [Hz]	Phenylephrine	3.9 ± 0.5	3.5 ± 0.6	***0.0151**	2.89	12
	Phenylephrine 115 s	4.0 ± 0.6	3.7 ± 0.7	0.091	1.88	11
	Isoprenaline	4.3 ± 0.5	4.3 ± 0.5	0.991	0.01	13
	Clonidine	4.9 ± 0.4	4.9 ± 0.5	0.801	0.26	11
Intraburst firing rate [Hz]	Phenylephrine	16.8 ± 1.7	16.2 ± 1.3	0.591	0.55	12
	Phenylephrine 115 s	16.9 ± 1.8	15.5 ± 1.0	0.341	1.01	11
	Isoprenaline	16.3 ± 1.1	15.4 ± 0.8	0.121	1.66	13
	Clonidine	14.4 ± 1.6	13.0 ± 1.0	0.98[2]	–	11
Coefficient of variation	Phenylephrine	0.72 ± 0.08	0.84 ± 0.10	****0.005**[1]	3.50	12
	Phenylephrine 115 s	0.69 ± 0.09	0.75 ± 0.09	0.12[1]	1.70	11
	Isoprenaline	0.63 ± 0.08	0.63 ± 0.09	0.68[3]	–	13
	Clonidine	0.46 ± 0.07	0.45 ± 0.07	0.71[1]	0.38	11

Bold values are to signify that these are statistically significant (< 0.05).
[1] Student's *t*-test for paired data.
[2] Mann–Whitney's *U* test.
[3] Wilcoxon's matched pair test.

Table 3. Effects of selective AR activation on bursting parameters in dopaminergic neurons

	Drug tested	Baseline	Drug administration	*p*	*t*	*n*
Bursting rate	Phenylephrine	0.5 ± 0.1	0.5 ± 0.1	0.58[1]	0.57	12
	Phenylephrine 115 s	0.4 ± 0.1	0.4 ± 0.1	0.921	0.10	11
	Isoprenaline	0.5 ± 0.1	0.4 ± 0.1	0.371	0.93	13
	Clonidine	0.4 ± 0.1	0.4 ± 0.1	0.91[2]	–	11
% of spikes in bursts	Phenylephrine	35.6 ± 6.7	38.9 ± 7.5	0.281	1.14	12
	Phenylephrine 115 s	32.4 ± 6.5	33.9 ± 5.7	0.731	0.35	11
	Isoprenaline	30.2 ± 6.9	29.4 ± 6.1	0.801	0.26	13
	Clonidine	21.2 ± 7.0	21.6 ± 8.4	0.632	–	11
Mean spikes per burst	Phenylephrine	3.2 ± 0.5	3.1 ± 0.3	0.572	–	12
	Phenylephrine 115 s	3.2 ± 0.5	3.1 ± 0.4	0.832	–	11
	Isoprenaline	2.8 ± 0.2	2.8 ± 0.2	0.881	0.16	13
	Clonidine	2.1 ± 0.3	3.3 ± 0.4	0.713	0.37	11
Mean intraburst interspike interval [ms]	Phenylephrine	66.5 ± 6.0	67.1 ± 5.9	0.972	–	12
	Phenylephrine 115 s	67.1 ± 6.6	68.8 ± 5.5	0.322	–	11
	Isoprenaline	65.6 ± 4.5	68.3 ± 3.8	0.141	1.59	13
	Clonidine	75.2 ± 6.3	80.0 ± 6.1	0.603	0.54	11
Mean burst length [ms]	Phenylephrine	159.7 ± 49.1	151.1 ± 30.0	0.472	–	12
	Phenylephrine 115 s	165.0 ± 53.5	156.7 ± 40.4	0.902	–	11
	Isoprenaline	126.3 ± 18.0	129.3 ± 18.4	0.792	–	13
	Clonidine	166.5 ± 30.1	186.4 ± 36.2	0.68[3]	0.43	11

[1] Student's *t*-test for paired data.
[2] Wilcoxon's matched pairs test.
[3] Student's *t*-test for unpaired data.

Table 1) had no effect on the total firing rate (baseline: 4.7 ± 0.5, IP: 4.6 ± 0.5, $n = 13$, n.s.; Fig. 5A; Table 2), bursting (baseline: 16.3 ± 1.1, IP: 15.4 ± 0.8; $n = 13$, n.s.; Fig. 5C; Tables 2 and 3), or extraburst activity of putative DAergic neurons (baseline: 4.3 ± 0.5, IP: 4.3 ± 0.5, $n = 13$, n.s.; Fig. 5B. Table 2). Similarly, the firing rate of putative non-DAergic neurons was not affected by the β-AR agonist (baseline: 5.2 ± 2.3, IP: 5.8 ± 2.3, $n = 13$, n.s.; Fig. 5D; Table 4). Additionally, no changes in coefficient of variation were observed both in cases of putative DAergic (baseline: 0.63 ± 0.08, IP: 0.63 ± 0.09, $n = 13$, n.s.; Table 2) and non-DAergic (baseline: 0.81 ± 0.10, IP: 1.02 ± 0.15, $n = 13$, n.s.; Table 4) neurons. However, changes in bursting parameters of non-DAergic neurons were observed. Both bursting rate (baseline: 0.12 ± 0.02, IP: 0.18 ± 0.02, $n = 13$, $p < 0.01$; Fig. 5G; Table 5) and% of spikes fired in bursts (baseline: 18.5 ± 4.4, IP: 27.2 ± 5.8, $n = 13$, $p < 0.05$; Fig. 5H; Table 5) were elevated while other bursting parameters were unchanged (Table 5). In summary, activation of β-AR with isoprenaline have not affected putative DAergic neurons' activity but increased bursting of non-DAergic neurons.

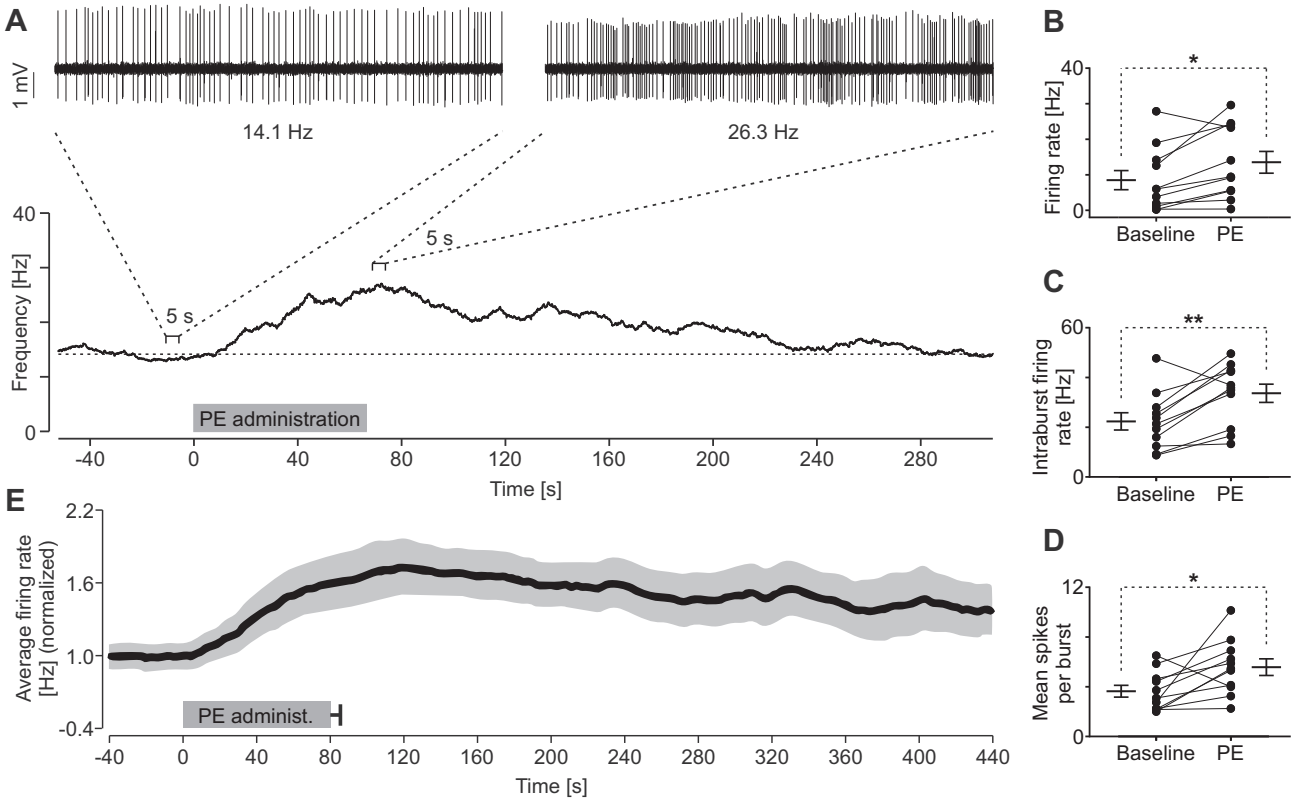

Fig. 3. Phenylephrine (α_1-AR agonist)-induced excitation of putative non-DAergic neurons in the VTA. (A) Representative neuronal activity before and after phenylephrine application. (B) Mean ± SEM and individual putative non-DAergic neurons' firing rates before (baseline) and after phenylephrine application (PE). (C) Mean ± SEM and individual putative non-DAergic neurons' intraburst firing rate before (baseline) and after phenylephrine application (PE). (D) Mean ± SEM and individual putative non-DAergic neurons' mean spike number per burst before (baseline) and after phenylephrine application (PE). (E) Average activity (normalized to baseline) of putative non-DAergic neurons in the VTA after local phenylephrine administration. Beginning of all time axes (0 s) was set to the beginning of phenylephrine application. Gray bars on A and E indicate drug application time. Lines or bars with whiskers on B, C, D and E indicate mean ± SEM. Black circles in B, C and D indicate individual neurons. **For $p < 0.01$, *for $p < 0.05$.

Table 4. Effects of selective AR activation on the firing rate of non-dopaminergic neurons

	Drug tested	Baseline	Drug administration	*p*	*t*	*n*
Total firing rate [Hz]	Phenylephrine	8.5 ± 2.7	13.5 ± 3.1	***0.0141**	2.96	11
	Isoprenaline	5.2 ± 2.3	5.8 ± 2.3	0.38[2]	–	13
	Clonidine	8.8 ± 1.7	7.7 ± 1.7	0.17[1]	1.61	6
Coefficient of variation	Phenylephrine	0.87 ± 0.14	0.84 ± 0.12	0.841	0.21	11
	Isoprenaline	0.81 ± 0.10	1.02 ± 0.15	0.101	1.80	13
	Clonidine	0.41 ± 0.07	0.54 ± 0.10	***0.0261**	3.13	6

Bold values are to signify that these are statistically significant (< 0.05).
[1] Student's *t*-test for paired data.
[2] Wilcoxon's matched pairs test.

DISCUSSION

Here, we show opposing effects of iontophoretically administered phenylephrine, a selective α_1-AR agonist, on firing of putatively DAergic and non-DAergic neurons recorded *in vivo* in the VTA of urethane anaesthetized rats. Phenylephrine application caused a relatively weak decrease in firing of putative DAergic neurons, whereas drug application induced a strong and prolonged excitation and increased bursting of putative non-DAergic neurons. In addition, application of selective α_2- or β-AR agonists (clonidine and isoprenaline, respectively) affected activity pattern, but not firing rate, of putative non-DAergic neurons while leaving activity of putative DAergic neurons intact.

Opposing effects of the iontophoretically applied α_1-AR agonist on firing of putative DAergic and non-DAergic neurons

Our results, showing phenylephrine-induced inhibition of putative DAergic neuronal activity in the VTA, are in line

Table 5. Effects of selective AR activation on bursting parameters in non-dopaminergic neurons

	Drug tested	Baseline	Drug administration	*p*	*t*	*n*
Bursting rate	Phenylephrine	0.16 ± 0.02	0.19 ± 0.03	0.18[1]	1.42	11
	Isoprenaline	0.12 ± 0.02	0.18 ± 0.02	**0.0041	3.53	13
	Clonidine	0.13 ± 0.02	0.12 ± 0.02	0.66[2]	–	6
% of spikes in bursts	Phenylephrine	21.5 ± 7.6	13.6 ± 3.8	0.102	–	11
	Isoprenaline	18.5 ± 4.4	27.2 ± 5.8	*0.0162	–	13
	Clonidine	5.7 ± 1.0	6.4 ± 0.9	0.541	0.66	6
Mean spikes per burst	Phenylephrine	3.6 ± 0.5	5.6 ± 0.7	*0.0231	2.69	11
	Isoprenaline	3.4 ± 0.5	3.3 ± 0.4	0.95[3]	–	13
	Clonidine	3.1 ± 0.5	3.6 ± 0.5	0.312	–	6
Intraburst firing rate [Hz]	Phenylephrine	22.4 ± 3.5	33.7 ± 3.7	**0.0031	3.83	11
	Isoprenaline	18.4 ± 3.7	20.3 ± 4.6	0.76[4]	0.31	13
	Clonidine	17.6 ± 2.2	19.2 ± 2.6	0.442	–	6
Mean burst interspike [ms]	Phenylephrine	57.5 ± 9.4	35.5 ± 5.5	**0.0052	–	11
	Isoprenaline	93.6 ± 21.9	99.7 ± 23.5	0.883	–	13
	Clonidine	63.0 ± 9.8	56.6 ± 6.7	0.442	–	6
Mean burst length [ms]	Phenylephrine	114.2 ± 7.9	139.2 ± 15.7	0.211	1.34	11
	Isoprenaline	152.0 ± 19.3	144.4 ± 17.9	0.784	0.29	13
	Clonidine	115.6 ± 14.5	132.7 ± 14.4	0.491	0.75	6

Bold values are to signify that these are statistically significant (< 0.05).

[1] Student's *t*-test for paired data.

[2] Wilcoxon's matched pairs test.

[3] Mann–Whitney's *U* test.

[4] Student's test for unpaired data.

with previous studies demonstrating that activation of α_1-AR within the VTA increases the frequency of sIPSPs in DAergic neurons and decreases their firing rate *in vitro* (Grenhoff et al., 1995; Paladini and Williams, 2004). In addition, our study is the first demonstration that local, iontophoretic application of phenylephrine decreases the activity of putative DAergic neurons *in vivo*. In contrast to our observations, a recent *in vivo* study demonstrated that intra-VTA pressure application of phenylephrine leads to excitation of putative DAergic neurons (Goertz et al., 2015). A number of possible explanations for the discrepancy in the results could be provided. The pressure drug application used by Goertz et al. (2015) might not be restricted to a single cell or small population of neurons, as spillover of the drug is inevitable. In addition, the administration pipette was located distally from the recorded neuron (~200–300 μm from the recording tip); therefore, phenylephrine could have presumably affected VTA circuitry as well as that of surrounding brain structures (Hupé et al., 1999). In contrast, the iontophoretic drug delivery used in the present study minimizes the possibility of observing spillover effects, as it is regarded to be restricted to the neuronal microcircuitry at the recording site (for review see: Lalley, 1999). In addition, it was demonstrated that α_1-ARs are expressed at the level of afferent fibers and terminals as well as dendrites within the VTA (Rommelfanger et al., 2009; Mitrano et al., 2012), leaving the possibility that the effects of α_1-ARs activation could vary depending on spillover of PE to inputs reaching dendrites of DA neurons. Pressure application of PE would more likely activate α_1-AR located on input fibers reaching the distal dendrites of DAergic neurons, as opposed to iontophoretic administration which is more likely to be restricted to the AR expressed at the proximity of the recording site. Thus, it is possible that the excitation observed by Goertz et al. (2015) is due to agonist reaching its target at more distant dendrites. Similarly, PE effects on the activity of DAergic neurons could critically depend on its dose, as inhibition of DAergic neurons was observed only with brief and excitation with prolonged PE application (Paladini and Williams, 2004). One might assume that phenylephrine concentration provided by pressure application in Goertz et al. (2015) should be higher and more prolonged than this of iontophoretic application in the present study. Therefore, it is possible that the excitatory effects were not observed in our study because PE dose was insufficient to desensitize the α_1-AR-mediated inhibition, as shown in the *in vivo* preparation (Paladini and Williams, 2004). Importantly, other data (*in vitro* electrophysiological recordings) obtained by Goertz et al. (2015) show a phenylephrine-induced increase in both the spontaneous-firing frequency (19.6%) and pharmacologically evoked burst firing frequency (13.9%) of the VTA DAergic neurons (Goertz et al., 2015). These data are further supported by electrochemical evidence, as intra-VTA infusion of prazosin, an α_1-AR selective antagonist, attenuated cocaine-induced release of DA in the NAc (Goertz et al., 2015). Importantly, other authors have also shown that *in vitro* activation of α_1-AR led to excitation of DAergic neurons through an increase in the frequency of glutamate-dependent sEPSCs and amplitude of evoked EPSCs (Velasquez-Martinez et al., 2012), as well as a decrease in the frequency of GABA-dependent sIPSCs and a reduction in the IPSC amplitude (Velasquez-Martinez et al., 2015). All of these observations suggest that activation of α_1-AR in the VTA may excite DAergic neurons through presynaptic mechanisms. Accordingly, Williams and colleagues demonstrated that NA can enhance glutamate release on DAergic neurons within the rostral linear

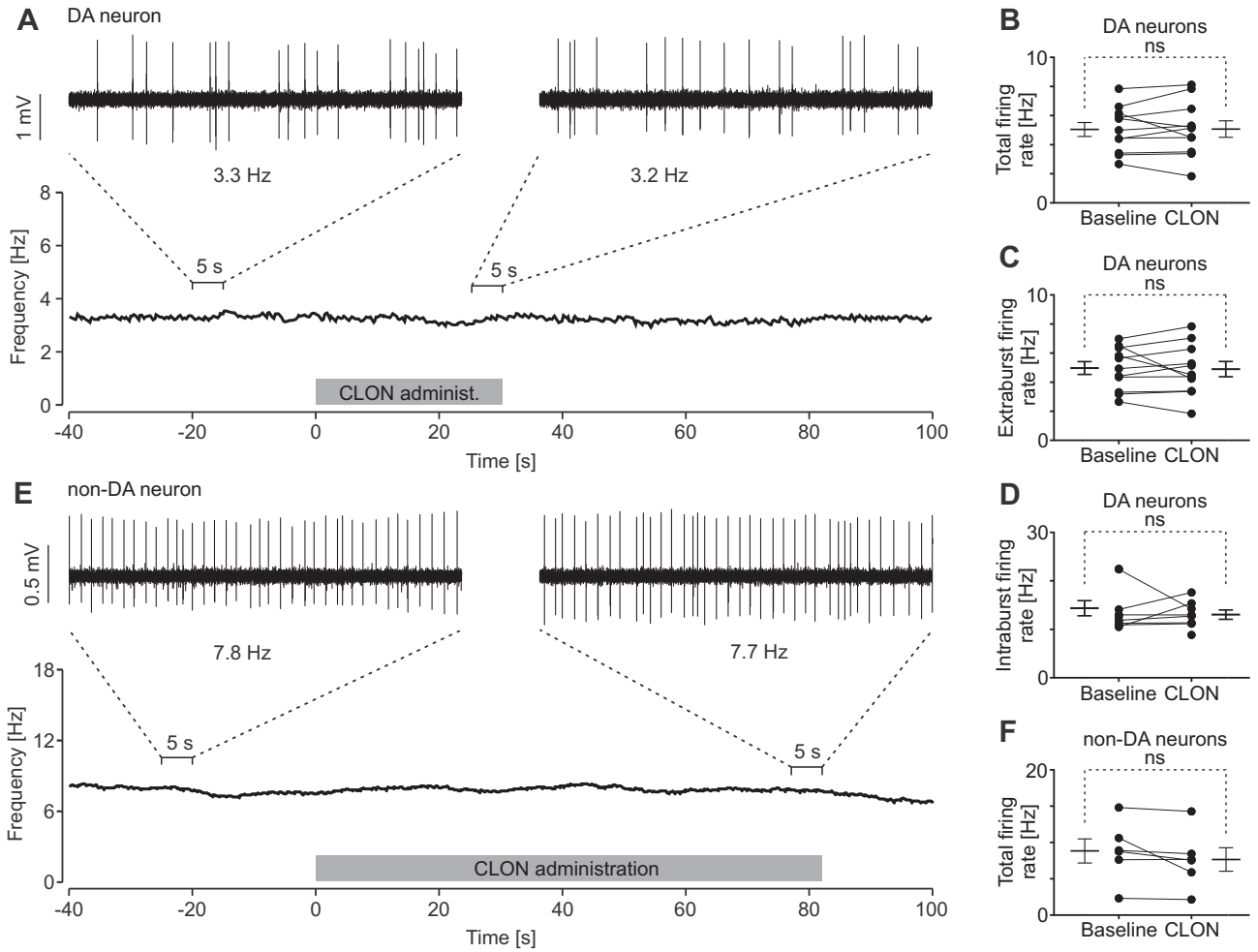

Fig. 4. Clonidine (α_2-AR agonist)-mediated effect on putative DAergic and non-DAergic neurons in the VTA. (A) Representative neuronal activity of putative DA neuron before and after clonidine application. (B) Mean ± SEM and individual putative DAergic neurons' firing rates before (baseline) and after clonidine application (CLON). (C) Mean ± SEM and individual putative DAergic neurons' extraburst firing rates before (baseline) and after clonidine application (CLON). (D) Mean ± SEM and individual putative DAergic neurons' intraburst firing rate before (baseline) and after clonidine application (CLON). (E) Representative neuronal activity of putative non-DAergic neuron before and after clonidine application. (F) Mean ± SEM and individual putative non-DAergic neurons' firing rates before (baseline) and after clonidine application (CLON). Beginning of all time axes (0 s) was set to the beginning of drug application. Gray bars on A and E indicate drug application time. Lines with whiskers on B, C, D and F indicate mean ± SEM. Black circles in B, C, D and F indicate individual neurons. ns: non-significant.

nucleus (RLi), a medial part of the VTA (Williams et al., 2014). Notably, in our study, the only neuron with DA-like electrophysiological properties that exhibited an increase in firing rate in response to iontophoretic phenylephrine administration was located in the RLi (data not shown); however, this cell was excluded from the final analysis due to the high probability of a false positive, as the physiological properties of DAergic and non-DAergic neurons in the medial VTA are similar and do not allow for a clear neuronal phenotype identification (Ungless and Grace, 2012; Yamaguchi et al., 2015). In contrast, the majority of putative DAergic neurons inhibited by phenylephrine in the present study were located in the lateral VTA, where DAergic and non-DAergic neurons are considered to display distinct electrophysiological properties (Grace and Bunney, 1980, 1983; Ungless and Grace, 2012). Moreover, our results do not preclude the possibility that higher PE dose could result in neurons' excitation. Unfortunately, higher iontophoretic currents introduced increased noise into the signal, preventing us from proper recordings of neuronal activity.

On the other hand, we observed that activating α_1-AR elicited a clear excitation of putative non-DAergic neurons. Such results are in line with previously reported phenylephrine effects *in vitro*, as α_1-AR activation in VTA slices led to excitation of non-DAergic neurons by decreasing the potassium conductance (Grenhoff et al., 1995). Our study is, to date, the first to demonstrate the effects of α_1-AR activation on VTA putative non-DA neurons *in vivo*. The observed enhancement in the firing rate was substantial (59% increase of the mean) and prolonged (peak in 115 s from the start of application), suggesting that α_1-AR activation leads to long-lasting changes in the membrane properties of putative non-DAergic neurons in the VTA. We also observed that intraburst firing rate was elevated and, accordingly,

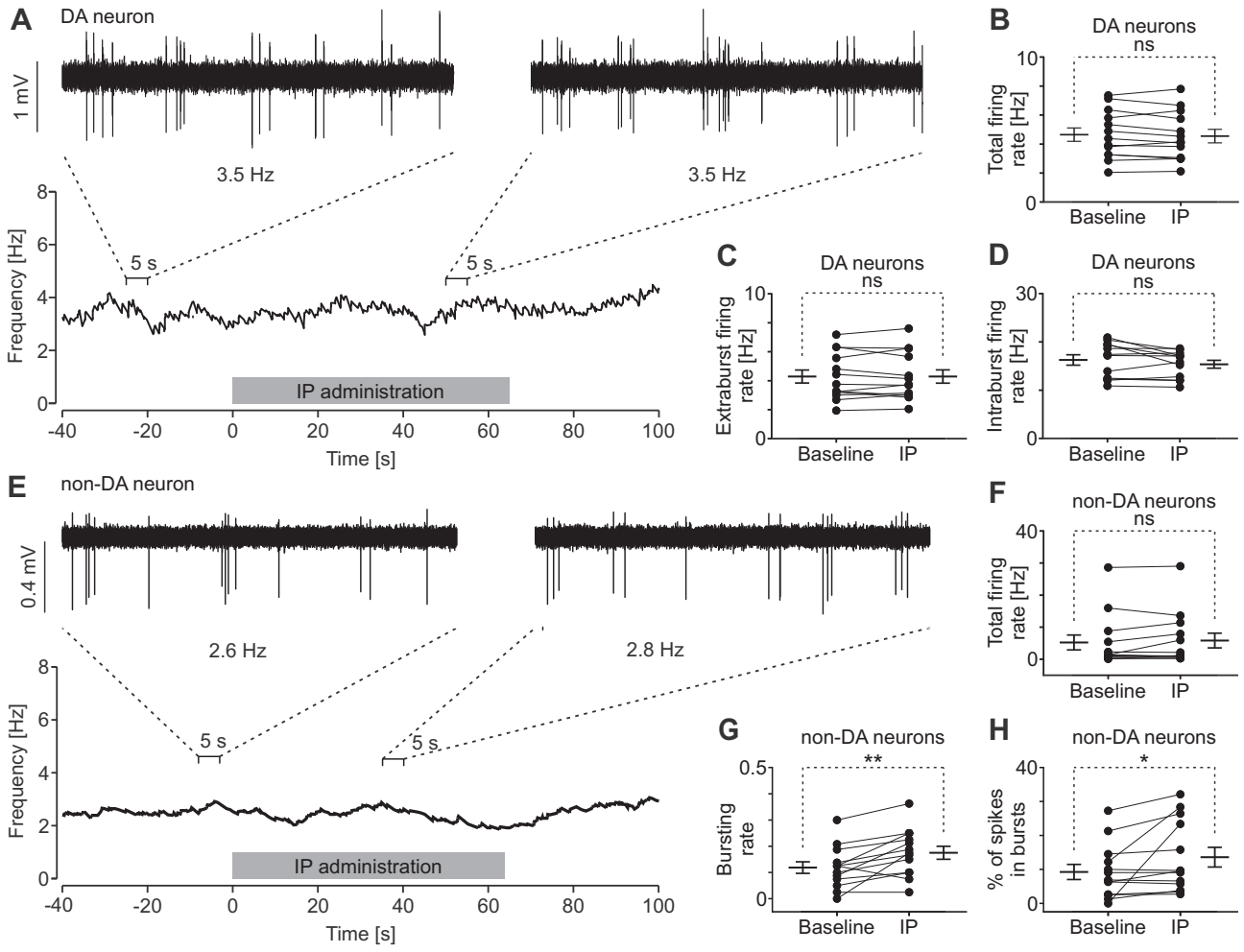

Fig. 5. Isoprenaline (β-AR agonist)-mediated effect on putative DAergic and non-DAergic neurons in the VTA. (A) Representative neuronal activity of putative DAergic neuron before and after isoprenaline application. (B) Mean ± SEM and individual putative DAergic neurons' firing rates before (baseline) and after isoprenaline application (IP). (C) Mean ± SEM and individual putative DAergic neurons' extraburst firing rates before (baseline) and after isoprenaline application (IP). (D) Mean ± SEM and individual putative DAergic neurons' intraburst firing rate before (baseline) and after isoprenaline application (IP). (E) Representative neuronal activity of putative non-DAergic neuron before and after isoprenaline application. (F) Mean ± SEM and individual putative non-DAergic neurons' firing rates before (baseline) and after isoprenaline application (IP). (G) Mean ± SEM and individual putative non-DAergic neurons' bursting rate before (baseline) and after isoprenaline application (IP). (H) Mean ± SEM and individual putative non-DAergic neurons' percent of spikes fired in bursts before (baseline) and after isoprenaline application (IP). Beginning of all time axes (0 s) was set to the beginning of drug application. Gray bars on A and E indicate drug application time. Lines with whiskers on B, C, D and F, G, H indicate mean ± SEM. Black circles in B, C, D and F, G, H indicate individual neurons. **For $p < 0.01$, *for $p < 0.05$, ns: non-significant.

mean burst interspike was attenuated after PE application. In addition, mean number of spikes per burst but not the burst length was significantly increased. These results indicate that activation of α_1-AR leads, apart from enhancement of the firing rate, to more potent and dense bursts of putative non-DAergic neurons. The putative non-DAergic neurons identified in our study could be considered most likely GABAergic, as cells synthesizing this neurotransmitter comprise more than 90% of non-DAergic neurons within the VTA (Nair-Roberts et al., 2008; Ungless and Grace, 2012). Additionally, the remaining non-DAergic neurons (glutamatergic) are located predominantly in the medial VTA (Yamaguchi et al., 2011, 2015; Morales and Root, 2014; Root et al., 2014), whereas our recordings were restricted to the central and lateral parts of the VTA. Thus, we hypothesize that α_1-AR activation in the VTA leads to an increase in GABAergic activity.

Altogether, our results allow us to put forward the hypothesis that the NAergic system can inhibit putative DAergic neuronal activity via activation of α_1-AR located on putative GABAergic cell bodies and/or terminals within the VTA. The observed phenylephrine-induced inhibitory responses of putative DAergic neurons were small and short-lasting compared to the strong, prolonged excitation of the putative GABAergic neurons elicited by direct phenylephrine application. This observation suggests that the inhibitory responses of the putative DAergic neurons observed during local application of phenylephrine could result from activation of α_1-AR on GABAergic terminals adjacent to the cell body of the recorded neuron. This possibility is further

supported by the fact that α_1-ARs are located presynaptically on GABAergic terminals within the VTA (Rommelfanger et al., 2009; Mitrano et al., 2012). In addition, the parameters of bursting activity of putative DAergic neurons observed in our study were not affected by local activation of α_1-AR. This observation suggests a modest decrease in putative DAergic neuronal excitability as has been reported by others (Grenhoff et al., 1995; Paladini and Williams, 2004). Reduced extraburst activity with unabated bursting of DAergic neurons could be interpreted as a noise reduction, leaving the neurons more permissive to phasic signal, as has been shown before (Almodovar-Fabregas et al., 2002; Velasquez-Martinez et al., 2012). Moreover, α_1-mediated enhancement of putative GABAergic neurons' bursts could account for the increased sensitivity of the VTA for potential salient stimuli. We cannot exclude the possibility that the weak inhibitory effects that we observed might also be mediated via α_1-AR located on the recorded putative DAergic neurons or by activating distant GABAergic somas. Both of these alternatives seem unlikely since activation of postsynaptic α_1-AR would preferably lead to excitation of putative DAergic neurons, whereas iontophoretic phenylephrine application minimizes the possibility to observe spillover effects.

Importantly, the proposed presynaptic mechanism does not preclude other effects of α_1-AR activation (i.e., increase in firing rate observed by the others), as the VTA DAergic neuronal responses could depend on the presence of GABAergic or glutamatergic terminals expressing α_1-AR, which might vary within the VTA DAergic subpopulations. Additionally, in the brain of anaesthetized animals, the balance between GABAergic and glutamatergic tone is shifted toward the inhibitory one (Hara and Harris, 2002). Thus, unlike in *in vitro* experiments, where the whole-brain network is not preserved, activation of α_1-ARs *in vivo* would augment stronger inhibitory rather than excitatory inputs to DAergic neurons.

Effects of iontophoretically applied α_2-AR agonist on firing of putative DAergic and non-DAergic neurons

Here, we show that iontophoretic clonidine application had no effect on neuronal activity in the VTA. Such results are in line with the previously described lack of effects of α_2-AR activation on putative DAergic neuronal activity *in vivo* (Aghajanian and Bunney, 1977; White and Wang, 1984) and *in vitro* (Grenhoff et al., 1995). On the other hand, activation of α_2-AR decreased glutamatergic-dependent EPSC frequency and sEPSC amplitude in the VTA DAergic neurons, as reported in some *in vitro* studies, suggesting attenuated glutamate release on DAergic neurons (Jimenez-Rivera et al., 2012; Williams et al., 2014). The α_2-AR agonist has also been demonstrated to decrease the firing rate of VTA DAergic neurons by lowering the hyperpolarization-activated cation current (I_h) *in vitro* (Inyushin et al., 2010). This discrepancy in the results between *in vivo* and *in vitro* experiments may represent inherent challenges in the interpretations of results obtained with those two methodological approaches. In accordance with the *in vivo* results mentioned above, we observed no α_2-AR-mediated effect on putative DAergic neurons. Moreover, our study is, to date, the first to investigate effects of α_2-AR activation on VTA putative non-DAergic neurons *in vivo*. The clonidine effects suggest that α_2-AR does not modulate firing rate of the VTA non-DAergic neurons, however makes firing slightly more irregular. Taking into account results from *in vitro* experiments (Inyushin et al., 2010; Jimenez-Rivera et al., 2012; Williams et al., 2014), α_2-AR-dependent modulation of VTA neuronal activity cannot be excluded; however, it was not observed in our *in vivo* study. Additionally, iontophoretic application has limited capacity of drug dosage, as higher currents and times can generate noise, thus preventing proper recording of neuronal activity. We used maximum iontophoretic current at which noise was not present, leaving the possibility that clonidine in these doses was insufficient to induce neuronal reaction, even though our observations are in line with the results obtained by others (Aghajanian and Bunney, 1977; White and Wang, 1984; Grenhoff et al., 1995).

Effects of iontophoretically applied β-AR agonist on firing of putative DAergic and non-DAergic neurons

The effects of β-AR activation at the level of the VTA have not been studied in detail so far. In an *in vitro* preparation, blocking and activating β-AR on midbrain DAergic neurons has been shown to have no effect on their activity (Grenhoff et al., 1995; Cathala et al., 2002; Williams et al., 2014). Concordantly, *in vivo* experiments have demonstrated weak or no effect on DAergic neuronal activity after β-AR agonist application and no effect after antagonist application (Aghajanian and Bunney, 1977; White and Wang, 1984). In line with those results, we found that iontophoretic isoprenaline application had no effect on the activity of putative DAergic neurons in the VTA. We also found that IP administration has no effect on firing rate of putative non-DAergic neurons. However, we have observed that β-AR activation significantly increased both bursting rate and percent of spikes occurring in bursts generated by putative non-DAergic neurons. Interestingly, we observed no inhibition of putative DAergic neurons after IP application, analogous to PE administration. Several explanations could be provided. The extent of bursting in putative non-DAergic neurons is relatively low, therefore elevation of bursting could not be strong enough to elicit significant impact on putative DAergic neuronal activity. Additionally, up to date there are no data regarding the presynaptic localization of β-ARs in the VTA, therefore IP administration on potential GABAergic terminals could not elicit any activity alteration of putative DAergic neurons. Moreover, mechanism of burst generation is rather restricted to soma of the cell, not the terminals, as to date there is no data demonstrating mechanism of burst generation at the terminals of the neuron. Importantly, this study is, so far, the first showing that local activation of β-AR influences the pattern of firing of VTA non-DAergic neurons. It was demonstrated in previous studies that pulsatile activity of VTA GABAergic neurons might be involved in controlling the pattern of activity of DAergic neurons (Paladini and Roeper, 2014; Morozova et al., 2016). Thus, demonstrated in this study

β-AR-mediated alteration of putative GABAergic neurons' bursting might contribute to overall NA-dependent functioning of DAergic system.

Limitations of the study

Our results highlight the role of AR signaling in the modulation of VTA activity; however, we should be cautious in the interpretation of the reported results with respect to the particular neurotransmission system. Despite the fact that the putative non-DAergic neurons recorded in our study can be plausibly classified as GABAergic, the exact phenotype was not determined (Grace and Bunney, 1983; Ungless and Grace, 2012). To confirm direct AR regulation of DAergic or GABAergic neuronal activity within the VTA, juxtacellular neuronal labeling or selective optogenetic light-driven activation should be performed. Furthermore, the VTA exhibits internal differentiation (Hnasko et al., 2012; Lammel et al., 2014; Yamaguchi et al., 2015), and our data are not sufficient to address the question about the subregional localization of AR activation responsive/non-responsive DAergic and non-DAergic neurons. Additionally, this study focuses on NAergic impact on spontaneous activity of VTA neurons, however different results could be observed in combination with stimulus or glutamate application, as demonstrated in other brain regions by others (Devilbiss and Waterhouse, 2000, 2002; Devilbiss et al., 2006). Despite those limitations, our research is a unique demonstration of the AR mechanisms involved in the modulation of VTA putative DAergic and non-DAergic neurons, providing receptor-specific bases for intra-VTA NAergic signaling *in vivo*.

CONCLUSIONS

Our results showed that VTA neuronal activity in the anaesthetized rat brain is preferentially modulated via α_1-AR selective agonist. We demonstrated that activation of α_1-AR *in vivo* evokes a modest inhibitory effect on putative DAergic neurons with no changes observed in bursting activity. In contrast, activation of α_1-AR led to a strong, prolonged excitation of putative non-DAergic neurons located in the VTA. We propose that NA acting primarily via α_1-ARs affects activity of the VTA by the regulation of DAergic and GABAergic neurons, with contrasting functional consequences. NA acting on α_1-AR located on GABAergic neurons' cell bodies and/or terminals increases VTA GABAergic tone, leading to inhibition of DAergic neuronal firing. We also demonstrated that activation of β-AR and α_2-AR, to a lesser extent, modulate the firing pattern of putative non-DAergic neurons. Thus NAergic modulation of VTA activity can involve mechanisms relying on various types of AR located on GABAergic neurons. However, physiologically occurring NA has some unique properties that cannot be mimicked by selective adrenergic receptor agonists. NA has been shown *in vivo* and *in vitro* to have profound potency to inhibit DAergic neurons via the D_2 receptor (Aghajanian and Bunney, 1977; White and Wang, 1984; Grenhoff et al., 1995; Arencibia-Albite et al., 2007; Guiard et al., 2008a). In addition, participation of α_2-AR in the inhibitory impact of NA on VTA neuronal activity has been suggested, as blocking these receptors attenuated the NA-induced inhibition of DAergic neurons *in vivo* (White and Wang, 1984; Guiard et al., 2008a), even though this effect was not confirmed by *in vitro* studies (Arencibia-Albite et al., 2007; Williams et al., 2014). Additionally, NAergic signaling might also facilitate the excitatory glutamatergic drive on DAergic neurons (Velasquez-Martinez et al., 2012; Williams et al., 2014) and responsiveness of DAergic neurons to glutamate (Almodovar-Fabregas et al., 2002) via an α_1-AR-dependent mechanism. Such a scenario might occur in a state of high activity of excitatory inputs to the VTA, such as during exposure to salient stimuli, potentially leading to an increase in phasic DAergic signaling (Goertz et al., 2015). Arguably, such a modulation may not be observed in an anaesthetized brain, when glutamatergic tone is attenuated. Therefore, NA can attenuate basal, extraburst activity of DAergic neurons, as described in our study (serving as a noise reducer), and at the same time, it can amplify excitatory inputs, as described by others (serving as a signal facilitator). These observations allow us to put forward the hypothesis that NA modulates VTA neuronal activity, leading to an increased signal-to-noise ratio of information encoded by DAergic neurons (Almodovar-Fabregas et al., 2002; Goertz et al., 2015), potentially increasing the value of important environmental stimuli.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

WBS and TB designed the experiments. KP performed the experiments, analyzed the results and performed the histological verifications. KP and WBS wrote the manuscript. WBS and TB reviewed the manuscript.

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