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Nicotine Modulation of the Lateral Habenula/Ventral Tegmental Area Circuit Dynamics: An Electrophysiological Study in Rats

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Abstract

Nicotine, the addictive component of tobacco, has bivalent rewarding and aversive properties. Recently, the lateral habenula (LHb), a structure that controls ventral tegmental area (VTA) dopamine (DA) function, has attracted attention as it is potentially involved in the aversive properties of drugs of abuse. Hitherto, the LHb-modulation of nicotine-induced VTA neuronal activity *in vivo* is unknown. Using standard single-extracellular recording in anesthetized rats, we observed that intravenous administration of nicotine (25-800 $\mu\text{g}/\text{kg}$ *i.v.*) caused a dose-dependent increase in the basal firing rate of the LHb neurons of nicotine-naïve rats. This effect underwent complete desensitization in chronic nicotine (6 mg/kg/day for 14 days)-treated animals. As previously reported, acute nicotine induced an increase in the VTA DA neuronal firing rate. Interestingly, only neurons located medially (mVTA) but not laterally (latVTA) within the VTA were responsive to acute nicotine. This pattern of activation was reversed by chronic nicotine exposure which produced the selective increase of latVTA neuronal activity. Acute lesion of the LHb, similarly to chronic nicotine treatment, reversed the pattern of DA cell activation induced by acute nicotine increasing latVTA but not mVTA neuronal activity. Our evidence indicates that LHb plays an important role in mediating the effects of acute and chronic nicotine within the VTA by activating distinct subregional responses of DA neurons. The LHb/VTA modulation might be part of the neural substrate of nicotine aversive properties. By silencing the LHb chronic nicotine could shift the balance of motivational states toward the reward.

Keywords: Electrophysiology, lateral habenula, dopamine, nicotine, ventral tegmental area, electrolytic lesion; extracellular recording.

Highlights

- Acute nicotine increases LHb neuronal activity in rats
- Chronic nicotine renders nicotine ineffective in modulating LHb neuronal activity
- Acute nicotine increases medial but not lateral DA VTA neuronal activity
- Chronic nicotine renders nicotine excitatory on lateral but not medial DA VTA neurons
- Similarly, LHb lesion renders nicotine excitatory on lateral but not medial DA VTA neurons

INTRODUCTION

The neural substrate responsible for both the rewarding and aversive effects of nicotine exposure is centered on the mesocorticolimbic dopamine (DA) system and involves several other brain regions and different neurotransmitter pathways (Di Matteo, Pierucci, Di Giovanni, Benigno, & Esposito, 2007). The nicotine effect is complex also considering that the ventral tegmental area (VTA) neurons are heterogeneous in both function and molecular identity (Morel et al., 2018; Pierucci, Chambers, Partridge, De Deurwaerdère, & Di Giovanni, 2014; H. Tan, Bishop, Lauzon, Sun, & Laviolette, 2009; K. R. Tan et al., 2012; van Zessen, Phillips, Budygin, & Stuber, 2012). The mechanisms likely to evolve after nicotine exposures to establish nicotine addiction are still not understood.

The lateral habenula (LHb) plays a pivotal role in modulating the DAergic function under physiological and pathological conditions such as drug addiction (Bianco & Wilson, 2009; Hikosaka, 2010; L. Lecourtier & Kelly, 2007). Different drugs of abuse known to induce aversive effects such as cocaine (Good et al., 2013; Jhou et al., 2013; W. Zuo, Chen, Wang, & Ye, 2013), alcohol (Wanhong Zuo et al., 2017) and nicotine (W. Zuo et al., 2016) increase LHb neuronal activity *in vitro* being part of the brain circuitry mediating CPA (Jhou et al., 2013; Wanhong Zuo et al., 2017) and condition taste aversion (CTA) (Glover, McDougle, Siegel, Jhou, & Chandler, 2016; Tandon, Keefe, & Taha, 2017) mediated by alcohol (Shiwalkar, Zuo, Bekker, & Ye, 2019). Moreover, acute and chronic nicotine-induced anxiety-like behavior is reversed by LHb lesion (Casarrubea et al., 2015). Both morphological and functional evidence demonstrated the existence of an indirect inhibitory control exerted by the LHb over VTA DA neuronal activity via the rostral-medial tegmental nucleus (RMTg) (Christoph, Leonzio, & Wilcox, 1986; Stephan Lammel et al., 2012; Lecca et al., 2011; Lecca, Melis, Luchicchi, Muntoni, & Pistis, 2012; Omelchenko & Sesack, 2007). However, there are no functional data *in vivo* illustrating how LHb neuronal activity is affected by acute and chronic nicotine exposure.

The classical DAergic theory of drug reward has been recently challenged. It is generally accepted that excitation of VTA DA and contextual DA release in accumbal and striatal terminals is the neural substrate of nicotine reward (V. Di Matteo, M. Pierucci, & E. Esposito, 2004; Nisell, Nomikos, & Svensson, 1994; M. Pierucci, V. Di Matteo, & E. Esposito, 2004; Schilström, Svensson, Svensson, & Nomikos, 1998). Nevertheless, it has been shown that the aversive effect produced by acute nicotine depends on an increase VTA DA signal, while the reward effect includes a GABA-dependent mechanism (Laviolette, Alexson, & van der Kooy, 2002; Laviolette & van der Kooy, 2004). The populations of DA neurons located in the lateral (latVTA) and medial VTA (mVTA) have been shown to represent different DA pathways mediating reward and aversion, respectively (Stephan Lammel, Ion, Roeper, & Malenka, 2011; S. Lammel, Lim, & Malenka, 2014; Stephan Lammel et al., 2012; Matsumoto & Hikosaka, 2009). Accordingly, it has been shown that medial VTA DA neurons showed increased population activity following presentation of aversive stimuli (Valenti, Lodge, & Grace, 2011) and increase of DA release in NAc shell, but not in the core (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Kalivas & Duffy, 1995). The ambivalent rewarding/aversive properties of nicotine could likely be related to functional sub-territories within the VTA themselves

modulated by the LHb for the “anti-reward” influence (Stephan Lammel et al., 2012). We postulate that nicotine, by acting in LHb, triggers distinct responses of latVTA and mVTA DA neurons that could favor rewarding circuits upon its chronic administration.

Using an *in vivo* electrophysiological approach, we first elucidated the effect elicited by the systemic administration of cumulative doses of nicotine (25-800 $\mu\text{g}/\text{kg}$ *i.v.*) on the basal neuronal activity of single LHb neurons in drug-naïve rats and then in rats treated with 6 mg/kg/day nicotine for 2 weeks. Using the same *in vivo* electrophysiological protocol, we studied the effect elicited by increasing doses of nicotine (25-800 $\mu\text{g}/\text{kg}$ *i.v.*) on single DA VTA neurons in naïve and chronic nicotine-treated animals or animals bearing a selective lesion of the LHb. The location of the recordings in the VTA was classically evaluated and revealed the distinct responses of the medial versus lateral subterritories of the VTA. The whole study indicates that the LHb plays an important role in mediating the effects of nicotine on the midbrain DA system by acting at different microcircuitries within the VTA.

Methods

Animals

Male Sprague-Dawley rats (250-350 g) were housed under a 12:12 hours light:dark cycle (lights on at 7:00 am) at a constant temperature of 21 ± 1 °C and relative humidity $60 \pm 5\%$ at the animal house of the Department of Physiology and Biochemistry, University of Malta. Food and water were available *ad libitum*. All experimental procedures were carried out in conformity with the European Law (EU Directive 2010/63/EU) and the Institutional Animal Use and Care Committee (IAUCC) at the University of Malta. All efforts were made to minimize animal pain and distress, as well as to reduce the number of experimental animals involved.

Extracellular single-unit recordings *in vivo*

Rats were anesthetized with an intraperitoneal (*i.p.*) injection of chloral hydrate (Sigma, UK; 8% w/v, 400 µg/kg, *i.p.*), mounted on a stereotaxic frame (model 1430, David Kopf Instruments, Tujunga, USA) and the skull was exposed. Throughout the experiment, the animal's body temperature was maintained at 37°C by a homoeothermic blanket system (Physitemp Instruments, Clifton, USA).

The activity of single neurons was recorded extracellularly using glass micropipettes filled with 2% Pontamine Sky Blue dye in 0.5 M CH₃COONa. The electrode was attached to a mechanical micromanipulator (SM-20, Narishige, Japan) and positioned over either the VTA or LHb using rat brain stereotaxic coordinates (see below). A burr hole was then drilled in the skull of the rats and, following the incision of the *dura mater*, the recording electrode was first lowered into the brain above the aforementioned structures and then slowly advanced using a one-axis hydraulic micromanipulator (MO-10, Narishige, Japan) until a spontaneously active (i.e., generating action potentials) neuron was found. Neural signals were amplified (Neurolog System, Digitimer Ltd, UK) with a bandpass filter set at 0.5–5 kHz, monitored through an oscilloscope (Tektronix Ltd, Bracknell, UK), digitalized and stored on a PC using a micro1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). Single unit activity was discriminated online using a voltage threshold and recorded as both continuous waveforms and events. After each experiment, the recording site was marked by the ejection of Pontamine Sky Blue dye (Sigma) from the electrode tip, achieved by applying a -10 µA current for 10 minutes to the electrode itself using an iontophoresis pump (BAB-501, Kation Scientific, Minneapolis, USA). The rat was sacrificed with an overdose of anesthetic and the brain was removed, washed in saline, and fixed by immersion in a 4% formaldehyde solution in saline for at least two days. To locate the dye spot, the brain was sectioned to 100 µm thick coronal slices using a vibroslicer (Campden Instruments, Loughborough, UK). Sections were mounted onto slides and visually inspected with the aid of a microscope. The location of the recording site, indicated by the blue dye spot (Fig. [1](#)), was then recorded on a rat brain diagram (see supplementary [figures-Fig. S1](#)).

Standard single-unit extracellular recording of LHb neurons was performed as described previously (Cristiano Bombardi et al., 2021; C. Bombardi et al., 2020; Delicata et al., 2018).

Micropipettes (4–7 M Ω resistance) were positioned in the LHB (set at a 10° angle, 3.4–3.8 mm anteroposterior from bregma, 1.4–1.8 mm mediolateral from the midline, 4–5 mm dorsoventral from the cortical surface) (Paxinos & Watson, 2007). The LHB spontaneously active electrophysiological characteristics were similar to what have been reported *in vivo* (Cristiano Bombardi et al., 2021; C. Bombardi et al., 2020; Delicata et al., 2018; Hartung, Tan, Temel, & Sharp, 2016), with an average firing rate of 16 Hz (0.5–39 Hz) and a waveform duration of > 1.0 ms (0.72 to 1.80 ms). Based on the pattern of spontaneous activity, neurons were classified as “tonic” (regular/irregular) or “bursty”. The majority of neurons (>95%) had a biphasic waveform (positive/negative), and an irregular firing pattern (>80%) (see Fig. S2).

Single DA neurons were recorded extracellularly *in vivo* from the VTA of the rats’ brain using the following stereotaxic coordinates (mm): 5.6–6.2 posterior to bregma, 2.0–1.6 lateral to the sagittal suture using a 10° angle of on the coronal plane, 7.0–8.5 ventral to the surface of the brain (Paxinos & Watson, 2007). Detected neurons were identified as DAergic according to well-established criteria (Bunney, Walters, Roth, & Aghajanian, 1973; Di Giovanni, Di Mascio, Di Matteo, & Esposito, 1998; Grace & Bunney, 1983; Ungless, Magill, & Bolam, 2004): triphasic/biphasic waveform showing a ‘notch’ on the positive rising phase, a duration \geq 1.1 ms from the start to the negative trough of the waveform, inhibitory response to a 15-second foot pinch, low pitch sound on the audio amplifier, inhibitory response to the injection of apomorphine and a relatively slow firing rate (1–10 Hz) characterized by the presence of bursts. Although these electrophysiological criteria have been extensively used, nonetheless, without direct neurochemical identification, the DAergic nature of recorded neurons cannot be known with certainty. Thus, from this point on, whenever VTA DA neurons will be mentioned, they have to be intended as ‘putative DA neurons’. Finally, bursts were identified based on well-established criteria (Grace & Bunney, 1984), defining the onset of a burst as the occurrence of an inter-spike interval < 80 ms and its termination by any following inter-spike interval > 160 ms. ~~As for the VTA, single LHB neurons were recorded extracellularly *in vivo* from the epithalamus of the rats’ brain using the following stereotaxic coordinates (mm): 3.6–4.2 posterior to bregma, 1.6–1.2 lateral to the sagittal suture using a 10° angle of on the coronal plane, 4.0–5.0 ventral to the surface of the brain.~~ For both the VTA and LHB, if no active cells were found within the set dorso-ventral range, the electrode was retracted back to the surface and moved to another track, usually located 0.2 mm more medial or caudal to the previous one, until a neuron was detected. When an active neuron was found, the baseline activity was recorded for at least 5 minutes before any drug treatment.

Drugs and administration protocols

(-)-Nicotine hydrogen tartrate salt ((-)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt, Sigma Aldrich, St.Louis, MO, USA), mecamylamine (N,2,3,3-Tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride, Abcam, Cambridge, UK) and (R)-(-)-apomorphine hydrochloride ((R)-5,6,6a,7-Tetrahydro-6-methyl-4H-dibenzo[de,g]quinoline-10,11-diol hydrochloride, Tocris/R&D Systems, Bristol, UK) were dissolved in saline, while haloperidol hydrochloride (4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone hydrochloride, Tocris/R&D Systems, Bristol, UK) was dissolved in a solution of acetic acid 10% (v/v) in saline. The pH was adjusted to about 7.4 if needed. The nicotine doses are reported as the salt (bitartrate) throughout the text. Therefore, the 6 mg/kg/day equals 2.1 mg/kg/day free base and the cumulative doses of nicotine (25 - 800 μ g/kg

i.v.) equals to 8.75-280 µg/kg i.v. free base These are low-medium doses of nicotine for rats (Matta et al., 2007). Mecamylamine is a non-selective, non-competitive nAChRs antagonist, apomorphine is a non-selective agonist to D₂-like receptors while haloperidol is a non-selective DA receptor antagonist.

Both LHb and VTA neurons were tested for their responses to the systemic administration of cumulative doses of nicotine (25-800 µg/kg) or an equal volume of saline (vehicle of nicotine). Drugs were injected intravenously (*i.v.*) through a lateral tail vein of the rat in a volume of 100 µl/0.3 kg for each injection. Nicotine cumulative doses (or equal volumes of saline) were injected at intervals of 2 min. Five min before either nicotine or saline administration, all rats were pre-treated with a single injection of saline (100 µl/0.3 kg), used as an internal control to account for any eventual effect of the injection, or mecamylamine (2 mg/kg, *i.v.*) in LHb neurons recorded from drug-naïve rats. In the case of VTA experiments, neurons were tested for their response to apomorphine (15 µg/kg, *i.v.*) and haloperidol (50 µg/kg, *i.v.*) at the end of each recording, to confirm their DAergic phenotype.

Two different groups of rats were used for the different experimental approaches. The drug naïve group (DN) was composed of rats that had never received nicotine before the experiment. Conversely, rats belonging to the chronic nicotine group (CN) were administered with 6 mg/kg/day of nicotine, split between three daily *i.p.* injections of 2 mg/kg each, for 14 consecutive days. On day 15, rats belonging to this group received a further challenge with cumulative doses of nicotine during the electrophysiology experiments, as described above. For all the experiments only one cell per animal was used.

LHb lesioning procedure

A group of 15 DN rats received an acute electrolytic lesion of the LHb (LHb Lesioned group) as previously shown (Casarrubea et al., 2015; Casarrubea et al., 2021), before starting the electrophysiological recording session of VTA DA neurons. Rats were anesthetized and fixed on a stereotaxic frame according to the experimental procedure described above (see 'Extracellular single-unit recordings in vivo' section). Two bipolar stainless steel stimulating electrodes (California Fine Wire, Grover Beach, CA, USA), with their tips exposed and separated by 0.5 mm, were attached to a micromanipulator, angled 10° to the coronal plane and lowered in the LHb using stereotaxic coordinates (3.6 mm posterior to bregma, 1.8 mm lateral to the midline, 5.0 mm ventral to the *dura mater* surface). A 500 µA current was applied for 30 seconds using a constant-current isolated stimulator (DS3 Digitimer, Hertfordshire, UK) to lesion the area. The second group of 15 rats received a sham lesion of the LHb (LHb Sham-Lesioned group), namely, they underwent the same procedure described for the LHb lesioned rats except no current was applied. The electrodes were usually left in place throughout the entire recording session and, in the case of LHb Sham-Lesioned rats, the localization of electrode tips was marked by passing a smaller current of 30 µA for just 3 seconds at the end of the experiment.

Both LHb Lesioned and Sham-Lesioned animals were tested and compared for their electrophysiological responses on VTA DA neurons to nicotine. Animals that underwent LHb lesion or sham-lesion were ~~killed~~ sacrificed at the end of the electrophysiology experiments by decapitation and the brains were removed. The extent of the lesions was then histologically

studied (Fig. S3). Lesions of the LHb were considered acceptable when surrounding regions (i.e., medial habenula, dorsal hippocampus and thalamic nuclei) were spared (see Fig. 1 and S3).

Off-line and statistical analyses of the data

Rate histograms were constructed by integrating discharge frequencies of single neurons over 10-sec epochs. The mean firing frequency (FR) was calculated for the baseline/pre-treatment periods and the 2 min intervals following each single drug administration (nicotine or saline). These values were normalized to the baseline and expressed as percentage change according to the following formula: $\left(\frac{FR_{drug} - FR_{baseline}}{FR_{baseline}}\right) \times 100$. All these normalized values were then averaged within each treatment group and summarized by dose-response. Values in all graphs are expressed as Mean \pm S.E.M..

With regards to the bursting activity of putative VTA DA neurons, spikes fired in bursts were identified as described above and expressed as a percentage of the total number of spikes detected (BR) for each interval considered. Bursting values for each interval were normalized to the baseline considering the absolute difference, defined as $\Delta = BR_{drug} - BR_{baseline}$. In the dose-response experiment, only the dose of 400 μ g/kg was considered for the BR. This dose was chosen on the basis that neurons usually showed the largest nicotine-induced increase of burst firing.

Data obtained from the dose-response curves were compared using a 1-way ANOVA for repeated measures (RM), considering the pharmacological treatment as the between factor and the cumulative doses as the within factor. RM-ANOVA with 1 RM factor time is equal to a 2-way ANOVA with subject*time. A normal 1-way ANOVA was used when more than two groups were compared without any within-subjects. The ANOVA was followed by the Fisher's PLSD post-hoc test, where appropriate.

All electrophysiological data were extracted, calculated and analyzed in Spike2 using a customized script. All statistical analyses were performed using Prism (v7, GraphPad Software Inc., La Jolla, CA, USA) and Stat View (SAS Institute, Cary, NC, USA). Significance was set at $p < 0.05$.

Results

Effect of the systemic administration of nicotine on the LHb neuronal activity of drug-naïve and chronic nicotine-treated rats

Systemic administration of nicotine caused a dose-dependent (25-800 µg/kg *i.v.*; $n = 13$) increase of the neuronal activity of the LHb neurons recorded in drug-naïve rats when compared to the control group ($n = 8$) ($F_{(3,33)} = 10.878$, $p < 0.0001$; Fig. 2a₁). The effect was already evident at the dose of 25 µg/kg, which enhanced the basal firing rate of the LHb cells by $16.7 \pm 8.7\%$. The maximum excitatory effect ($75.4 \pm 17.1\%$) was observed after the administration of 200 µg/kg. Administration of higher doses caused a lower level of excitation with the 800 µg/kg inducing only a $38.2 \pm 16.6\%$ increase. A representative rate histogram is reported in Fig. 2a₂, which shows the typical excitatory effect of nicotine. In contrast, mecamylamine injection (2 mg/kg, *i.v.*) did not cause any change in firing rate ($n = 8$; $p = 0.981$ Fig. 2a₃) but prevented nicotine-induced excitation of the LHb neurons ($n = 8$) ($F_{(3,33)} = 10.878$, $p < 0.0001$) when compared to saline pre-treatment ($n = 8$) (Fig. 2a₁, 2a₃). The mean basal firing rate of the different groups was not different from the average mean of all the neurons recorded (16.7 ± 1.9 Hz). Finally, the localization of recorded neurons was reconstructed histologically and plotted on a rat brain diagram of the LHb (Fig. S31B). Recording locations spread throughout the entire extension of the LHb and no preferential distribution was observed for nicotine-induced responses (data not shown Fig. S4).

Unlike in drug-naïve animal, dose-response administration of nicotine (25-800 µg/kg *i.v.*; $n = 7$) did not cause any change in the firing rate of the LHb neurons recorded in chronic nicotine (6 mg/kg/day for 14 days) treated rats when compared to the effect of saline administration ($n = 7$) ($F_{(1,12)} = 0.117$, $p = 0.738$; Fig. 2B). The maximum change in firing ($6.8 \pm 19.8\%$) was observed after the administration of 800 µg/kg. Two representative rate histograms are reported in Fig. 2b_{2,3}, which show the typical lack of effect of nicotine in chronic nicotine-treated rats. The chronic nicotine treatment did not change the basal firing rate of LHb neurons when compared to drug naïve rats (16.2 ± 1.7 Hz and 17.1 ± 2.3 Hz, respectively). Moreover, the localization of recorded neurons was reconstructed histologically in the same way as the drug-naïve group. Most of the recorded neurons were localized in the medial subdivision of the LHb and showed an almost homogeneous response of the LHb neuronal population to the challenge with nicotine following its chronic administration (data not shown).

Effect of the systemic administration of nicotine on the VTA DA neuronal activity of drug-naïve rats

Acute treatment with nicotine (25-800 µg/kg, *i.v.* $n = 12$) induced a dose-related increase in the firing activity of DA neurons in the VTA when compared to saline administration ($n = 8$; Fig. 3) ($F_{(1,18)} = 4.615$, $p < 0.05$). The maximal increase of the firing rate was reached after the administration of 800 µg/kg which enhanced the activity of DA neurons by $29.1 \pm 12.0\%$ (above baseline) (Fig. 3a₁). Two representative rate histograms are reported in Fig. 3a₂ and 3a₃ showing the lack of effect of saline administration and the increase in firing rate induced by

acute nicotine, with the typical biphasic (excitation/inhibition) response induced by the higher doses of the drug.

The VTA DA neurons were classified as either latVTA or mVTA neurons, depending on their anatomical localization (Fig. S1). When recorded neurons were split according to their location within the VTA (Fig. 3B,C), latVTA neurons ($n = 6$) were not affected ($F_{(1,8)} = 0.117$, $p = 0.741$; *vs* saline $n = 4$. Fig. 3b_{1,2}) while VTA mVTA DA neurons ($n = 6$) were excited by $46.7 \pm 14.6\%$ ($F_{(1,8)} = 14.584$, $p < 0.01$; *vs* saline $n = 4$ Fig. 3c_{1,2}) above baseline at the dose of 800 $\mu\text{g}/\text{kg}$. With regard to the burst firing, nicotine did not significantly increase the number of bursts when all neurons were pooled together (Unpaired t-Test, $t_{18} = 1.404$, $p = 0.177$). However, a significant nicotine-induced increase in burst firing was found for mVTA but not for latVTA neurons (Unpaired t-Test; mVTA $t_8 = 2.407$, $p < 0.05$; latVTA $t_8 = 0.311$, $p = 0.764$), as previously described for the firing rate (data not shown).

Effect of the systemic administration of nicotine on the VTA DA neuronal activity of chronically nicotine-treated rats

Following a 14-day chronic nicotine treatment, VTA DA neurons recorded from non-lesioned rats were tested on day 15 with a further challenge of either nicotine, administered systemically in cumulative doses (25 - 800 $\mu\text{g}/\text{kg}$ i.v., $n = 16$), or an equal volume of saline (control group, $n = 13$). When all neurons were pooled together, the acute challenge with nicotine failed to significantly increase the basal firing rate ($F_{(1,27)} = 1.444$, $p = 0.240$; Fig. 4 a₁) and burst firing (Unpaired t-Test, $t_{27} = 0.002$, $p = 0.998$; not shown) activities of recorded neurons. Two representative rate histograms are reported in Fig. 4a₂ and a₃ showing the lack of effect of both nicotine and saline administration.

When DA neurons were classified according to their localization within the VTA (Fig. 4B,C), neurons in the latVTA showed the strongest dose-dependent response to nicotine administration ($F_{(1,12)} = 7.172$, $p < 0.05$; NIC $n = 6$, SAL $n = 8$; Fig. 4b_{1,2}) that reached the mean value of $33.6 \pm 19.9\%$ above baseline at the dose of 800 $\mu\text{g}/\text{kg}$. Conversely, nicotine-induced response in mVTA neurons did not differ from controls ($F_{(1,13)} = 0.127$, $p = 0.727$; NIC $n = 10$, SAL $n = 5$; Fig. 4c_{1,2}).

Finally, nicotine did not significantly change basal burst firing of both latVTA and mVTA (Unpaired t-Test, respectively: $t_{12} = 0.051$, $p = 0.960$, $t_{13} = -0.015$, $p = 0.988$; data not shown).

Effect of the systemic administration of nicotine on the VTA DA neuronal activity of drug-naïve rats following the LHb lesion

The LHb was either lesioned ($n = 15$) or sham lesioned ($n = 15$) in two more groups of drug-naïve rats. The effect induced by the injection of cumulative doses of nicotine was then compared to the two previous groups of non-lesioned rats, that received either nicotine ($n = 12$) or saline (control, $n = 8$; Fig. 5a). When all neurons were pooled together, no significant differences were observed between the four different groups ($F_{(3,46)} = 1.573$, $p = 0.209$; Fig. S4S5).

However, when the analysis was split by the two VTA subnuclei, latVTA neurons, that did not respond to nicotine in the non-lesioned ($n = 6$) and sham-lesioned ($n = 10$) groups when

compared to saline ($n = 4$), showed a significant increase in their firing rate following the lesion ($n = 4$) of the LHB ($F_{(15,100)} = 3.046$, $p < 0.01$; Fig. 5A). Conversely, the nicotine-induced increase in firing rate, observed for mVTA neurons in non-lesioned rats ($n = 6$), was almost completely abolished by the LHB-lesioned group ($n = 11$) ($F_{(15,110)} = 3.696$, $p < 0.01$; Fig. 5B). Examples of the effects produced by nicotine on PBN and mVTA neurons recorded in LHB sham and lesioned rats are shown in Fig. 5a_{1,2} and b_{1,2}.

As for the bursting activity, there were no significant differences in nicotine-induced changes in burst firing between non-lesioned, sham-lesioned, lesioned or saline groups for all neurons pooled together ($F_{(3,49)} = 1.735$, $p = 0.173$), as well as for latVTA ($F_{(3,23)} = 1.066$, $p = 0.386$) and mVTA ($F_{(3,25)} = 1.839$, $p = 0.170$) neurons (data not shown).

Discussion

The present study shows that acute intravenous nicotine administration enhances the basal firing rate of the LHb neurons of chloral hydrate anesthetized rats by activating nAChRs. Strikingly, we showed for the first time that chronic nicotine treatment prevented nicotine-induced LHb excitation. Moreover, we further confirm recent evidence showing that nicotine induces different responses in distinct populations of VTA DA neurons. We found that acute nicotine produced excitation in mVTA DA neurons and had no effect on latVTA cells. Following chronic nicotine exposure, this pattern of nicotine effects was inverted with an increase of latVTA neuronal activity and no effect in mVTA. Lesion of the LHb similarly to chronic nicotine, switched the effect of acute nicotine in the VTA, increasing the activity of latVTA neurons while being ineffective on mVTA cells.

Our findings of the excitatory nicotine control of LHb activity are not only consistent with previous evidence showing that nicotine increases LHb neuronal activity *in vitro* (W. Zuo et al., 2016) but also extend it showing that this excitatory nicotine-modulation of the LHb also occurs *in vivo* at a concentration achieved by smokers (Matta et al., 2007). Nicotine increases the activity of LHb neurons *in vitro* through direct depolarization, via postsynaptic $\alpha 6$ -containing $\alpha 6$ *nAChRs but also modulating GABA and GLU input onto LHb neurons (W. Zuo et al., 2016). Nevertheless, the nicotine effects that we recorded in the LHb *in vivo* may also be dependent on the activity of many areas projecting to the LHb such as VTA neurons (Yoo et al., 2016) and dorsal and medial raphe neurons (reviewed in (Metzger et al., 2021) known to be affected by nicotine.

The loss of reactivity of LHb neurons to nicotine in chronic nicotine-treated rats is one of the major findings of our study. Chronic nicotine induces neuroadaptations such as changes in nAChR expression, synaptic plasticity, anatomical and behavioral changes that lead to both tolerance and sensitization phenomena to nicotine (Di Matteo et al., 2007). A dramatic sensitization of the medial portion of the habenula (MHb) and its interpeduncular nucleus pathway has been described due to exposure to chronic nicotine (Arvin et al., 2019) elicited by $\alpha 5$ *nAChRs that seem to mediate the inhibitory effects of higher nicotine doses on brain reward systems (Fowler, Lu, Johnson, Marks, & Kenny, 2011). A plausible candidate to explain the desensitization of the LHb neurons to nicotine might be the alteration of AChR signaling within this area, nevertheless, we did not observe any change in $\alpha 7$ *nAChRs $\alpha 4$ *nAChR protein expression in the LHb following chronic nicotine (Supplementary information, Fig. [S5S6](#)). Moreover, neuroadaptation of other neurotransmitter systems cannot be excluded. For instance, we have recently shown that the serotonergic 5-HT_{2A}R and the 5-HT_{2C}R signals are profoundly altered after chronic exposure to an identical nicotine treatment mainly in the LHb (Cristiano Bombardi et al., 2021; C. Bombardi et al., 2020). Our findings on nicotine-modulation of the LHb neuronal activity in chronic nicotine-treated rats together with previous observations further support a pivotal involvement of this area in the drug of addiction brain circuitry, and notably toward DA neurons of the VTA.

The distinct responses of DA neurons to acute nicotine are in line with recent evidence showing that DA cell response to nicotine is more heterogeneous than previously thought (Eddine et al.,

2015; Mameli-Engvall et al., 2006; Nguyen et al., 2021). Indeed, it has been shown that nicotine (both 30 µg/kg single bolus or 0-90 µg/kg dose-response) i.v. excited latVTA neurons projecting to NAc while inhibited mVTA neurons projecting to the amygdala (Amg) and these two populations were similar in terms of electrophysiological properties *in vivo* in mice (Eddine et al., 2015; Nguyen et al., 2021). Accordingly, nicotine injection increased NAc DA release and decreased Amg DA release (Nguyen et al., 2021). Interestingly, optogenetic inhibition of Amg-projecting mVTA DA neurons was anxiogenic, while photoactivation activation of NAc-projecting latVTA DA neurons was rewarding, mimicking the anxiety and reward, respectively induced by general nicotine administration in mice (Nguyen et al., 2021). In line with this study, we observed two main nicotine effects that according to the anatomical localization of the VTA DA neurons recorded but not their electrophysiological characteristics. Conversely to the recent evidence in mice, we observed an opposite pattern of effects of nicotine on VTA neurons, with a prevalent nicotine-induced excitation of mVTA neurons while latVTA neurons were unaffected. The reason for the difference between our and Faure and colleagues' studies (Eddine et al., 2015; Nguyen et al., 2021) is difficult to explain, it might be due to the different species used (rats vs mice) and different circuitries involved or technical reasons. In rats, the only inhibitory transient effect (followed by excitation) reported after nicotine administration was seen in a VTA DA population presenting firing slow oscillations of their firing rate (D. Zhang et al., 2012). Nevertheless, we have never observed this nicotine's initial inhibitory effect. The robustness of our data that the effects of nicotine are segregated according to the VTA territories is further supported by the finding that the lesion of the LHb induced the opposite profile of responses of DA neurons to acute nicotine. Indeed, latVTA neurons were now stimulated whereas mVTA neurons were unaltered by nicotine. This result highlights that LHb plays a role in the regional pattern of VTA neuron response to acute nicotine, a role that is likely lost in chronic nicotine conditions since we showed that LHb is no longer responding to nicotine under this condition. Nevertheless, considering that the LHb lesion increases DA release in the prefrontal cortex, NAc and dorsolateral striatum (Lucas Lecourtier, Defrancesco, & Moghaddam, 2008) and 5-HT turnover in the dorsal raphe nucleus (Yang, Hu, Xia, Zhang, & Zhao, 2008) an indirect effect for explaining the findings of this study cannot be ruled out.

Several plastic adaptations of the DAergic system are elicited by nicotine chronic exposure. Indeed, the reward signal induced by chronic nicotine switches from a DA-independent to a DA-dependent neural system (H. Tan et al., 2009). In chronic-nicotine-treated animals, a dose of nicotine capable of inducing CPA when infused into the VTA in nicotine-naïve rats produced instead conditioned place preference (CPP), both reverted by DA receptor blockade (H. Tan et al., 2009).

We did not observe any changes in the basal firing rate of the recorded VTA DA neurons after the LHb lesion. On the other hand, a slight increase of their firing rate and an impairment of the positive reward predicting error coding in DA neurons was described after LHb lesion in mice (Tian & Uchida, 2015). Moreover, we did not find any changes in the firing rate of the VTA DA neurons recorded in chronically-treated rats with nicotine 6 mg/kg/day when compared to control rats in agreement with the lack of effect of 2.4 mg/kg/day nicotine treatment (Besson et al., 2007). Nevertheless, both an increase of firing rate and burst firing of

VTA DA neurons after oral nicotine administration (200 µg/ml for 19 days) (Morel et al., 2018) and a decrease of their basal firing rate after 9 mg/kg/day i.p. (H. Tan et al., 2009) have been reported. Interestingly, this latter study showed nicotine challenge induced a stronger excitation of VTA DA neurons while it was ineffective in increasing VTA GABA neuron firing rate in animals chronically treated with nicotine compared to drug-naïve animals (H. Tan et al., 2009), an effect that involved endocannabinoid 2-arachidonoylglycerol (Buczynski et al., 2016). Loss of this inhibitory feedback mechanism likely contributes to increased VTA sensitivity to nicotine and other stimuli (Johnson, Blomqvist, Engel, & Söderpalm, 1995; Vihavainen et al., 2008), resulting in sensitized DA release in the nucleus accumbens (Vincenzo Di Matteo, Massimo Pierucci, & Ennio Esposito, 2004; Zhang, Dong, Doyon, & Dani, 2012) and increased motivation for nicotine intake.

Our present experiments in chronic nicotine-treated animals show instead a decrease in sensitivity to systemic nicotine compared to controls in agreement with recent evidence showing chronic nicotine treatment can markedly impair DA neuron activation in response to a subsequent intravenous nicotine injection (Morel et al., 2018). On the other hand, no change in sensitivity of the VTA DAergic system in chronically treated animals has also been reported (Vincenzo Di Matteo et al., 2004; Pierucci et al., 2014; Massimo Pierucci, Vincenzo Di Matteo, & Ennio Esposito, 2004). An explanation for these discrepancies in sensitivity to nicotine after chronic treatment is difficult to find, although earlier studies (Morel et al., 2018; H. Tan et al., 2009) did not differentiate the VTA neurons according to their locations and therefore the experimenters might have casually recorded from those cells that were differently affected by nicotine. Consistent with the view that nicotine-induced aversion in drug naïve animals might be driven by the LHb, we have recently shown that nicotine-induced anxiety-like behavior and temporal-pattern behavioral reorganization are both reversed by the selective LHb lesion in rats (Casarrubea et al., 2015; Casarrubea et al., 2021). Moreover, in line with these observations, cocaine-induced LHb activation mediates CPA (Jhou et al., 2013; W. Zuo et al., 2013) and alcohol-induced LHb activation mediates condition taste aversion (Glover et al., 2016; Tandon et al., 2017) that is reversed by LHb lesion (Shiwalkar et al., 2019). The nicotine aversion conveyed by LHb would be dependent on halting DA latVTA neurons projecting to NAc and mediating reward by the LHb-RMTg-VTA circuitry, and directly activating LHb-mVTA neurons, mediating aversion and projecting to the prefrontal cortex (Stephan Lammel et al., 2012; Stamatakis & Stuber, 2012) (see Fig. 6A). Under chronic nicotine exposure, the LHb is silenced (as in LHb lesioned animals) thereby switching the activation of the subpopulations of VTA DA neurons with latVTA neurons; the increased firing of these latVTA neurons could participate to the positive reward properties of chronic nicotine (Fig. 6B).

Our data fit with the evidence that the aversive effect produced by acute nicotine depends on an increase VTA DA signal, while the reward effect includes a DA-independent mechanism (Laviolette et al., 2002; Laviolette & van der Kooy, 2004). Nevertheless, the scenario is more complicated since, at least in mice, some mVTA decrease their input to the Amg (Nguyen et al., 2021) contributing to nicotine aversive properties. Interestingly, an LHb-Amg circuitry has been described in mice (Zhou et al., 2019), although other anatomical investigations fail to report it (e.g., (Zahm & Root, 2017)), so it would be important to confirm this in the rat.

Our study has some obvious limitations. For instance, all *in vivo* electrophysiological data were recorded under chloral hydrate anesthesia. Therefore, we cannot directly draw any conclusions about reward/aversion nicotine-mediated behavior and relevant activity patterns in DA neurons subpopulations. Repeating the study in freely-moving animals, although challenging, is pivotal to testing this hypothesis with contextual use of voluntary intake instead of experimenter-administered nicotine. Finally, we recorded putative DA neurons identified based on their electrophysiological characteristics and pharmacology (Bunney et al., 1973; Grace & Bunney, 1983, 1984) but they were not immunocytochemically confirmed DA neurons. Because DA, GABA and GLU neurons have been described within the VTA (Dobi, Margolis, Wang, Harvey, & Morales, 2010; S. Lammel et al., 2014; Matsumoto & Hikosaka, 2009; K. R. Tan et al., 2012; van Zessen et al., 2012) and that these cells express functional nAChRs (Calabresi, Lacey, & North, 1989; Mansvelder, Keath, & McGehee, 2002; Yijin Yan, Beckley, Kim, & Drenan, 2019; Y. Yan et al., 2018), the inclusion of some non-DA neurons among the cells recorded here cannot be completely excluded. We used 6 mg/kg/day of nicotine bitartrate (corresponding to 2.1 mg/kg/day free base) for chronic experiments. This is the typical dose delivered in animal models of nicotine exposure that gives nicotine levels similar to those detected in moderate to heavy smokers (Matta et al., 2007). Nevertheless, more doses of nicotine should be tested to see how the LHb/VTA dynamics would change. Finally, our experiments were performed in male rats but a growing number of studies reports gender-specific effects of nicotine that need to be addressed in future studies (Chellian et al., 2021; Cruz et al., 2021; O'Dell & Torres, 2014).

Previous reports (Mansvelder et al., 2002; Mansvelder & McGehee, 2000; H. Tan et al., 2009) have shown that chronic nicotine induces a functionally relevant switch in the motivational signaling properties of nicotine, by enhancing motivational and appetitive salience of nicotine during the addiction process. Thus, our findings support this novel perspective further suggesting that the LHb might be responsible for the described switch in the motivational valence of nicotine. This evidence could facilitate the development of compounds capable of reverting the hypofunctionality of the LHb observed in the chronic nicotine state, with the tentative aim of smoking cessation.

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Author Contributions

Conceptualization, M.P. and G.D.G.; methodology M.P., G.D.G.; formal analysis, M.P., F.D., and G.D.G.; resources, G.D.G.; data curation, M.P., A.M.G., F.D., R.C., M.C., A.P., P.D.D., and G.D.G.; writing—original draft preparation, M.P., P.D.D., and G.D.G.; writing—review and editing, all authors; supervision, G.D.G.; project administration, G.D.G.; funding

acquisition, G.D.G.. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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FIGURE 1

Histological confirmation of the recording/lesioning site localization.

Histological confirmation of the recording site localization. (A) Rat brain diagrams showing the localization of the LHb and VTA on both sagittal and coronal planes. (B) Optical microscope photograph showing an example of a coronal slice containing the LHb with the Pontamine Sky Blue dye spot (white arrowhead), confirming the localization of the recording electrode tip. (C) The picture shows a coronal diagram and photomicrograph of the rat VTA, illustrating the localization of the blue dye spot within this area (red arrowhead). (D) Photomicrograph of a coronal section of the rat brain containing the LHb and showing its electrolytic lesion. **DG** Dentate Gyrus, **SM** Stria Medullaris, **fr** Fasciculus Retroflexus, **VTA** Ventral Tegmental Area, **LHbm** Medial Division of the LHb, **LHbl** Lateral Division of the LHb, **MHb** Medial Habenula, **3V** Third Ventricle, **PV** Paraventricular Nucleus of the Thalamus, **PBN** Parabrachial Pigmented Nucleus (latVTA), **PN** Nucleus Paranigralis (mVTA), **SNe** Substantia Nigra pars compacta, **SNr** Substantia Nigra pars reticulata, **IF** Interfascicular Nucleus, **IP** Interpeduncular Nucleus, **ml** Medial Lemniscus, **mp** Medullary Peduncle.

FIGURE 2

Nicotine induces a dose-dependent increase of LHb neuronal activity in drug-naïve rats but not in rats chronically treated with nicotine.

(A) Systemic administration of nicotine dose-dependently increased the neuronal activity of single LHb neurons in drug-naïve rats. For each neuron, following a baseline recording period, either mecamylamine (2 mg/kg, *i.v.*) or saline (vehicle; 100 μ l, *i.v.*) were administered as a pre-treatment. Cumulative doses of nicotine (25 - 800 μ g/kg *i.v.*) were administered 5 min after pre-treatment. Mean firing rate values calculated following each injection were normalized to the basal mean activity and expressed as percentages. (a₁) Dose-response curves showing the effect of nicotine administration (**VEH+NIC**, n = 13) compared to saline (**VEH+SAL**, n = 8), following a pre-treatment with either mecamylamine (**MEC+NIC**, n = 8) or its vehicle (**VEH+SAL**, n = 8). Nicotine significantly increased the basal firing rate of LHb neurons compared to its saline controls. This effect was completely prevented by mecamylamine pre-treatment (1-way ANOVA rm followed by Fisher's PLSD post-hoc test; * $p < 0.05$, ** $p < 0.01$ vs VEH+SAL; ° $p < 0.05$, °° $p < 0.01$ vs MEC+SAL; ### $p < 0.01$ vs MEC+NIC). The rate meters show an example of the nicotine-induced increase of basal firing frequency (a₂) and its antagonism by mecamylamine pre-treatment (a₃). (B) Chronic nicotine treatment abolished the response induced by acute nicotine administration. (b₁) The dose-response graph shows the average response of LHb neurons (n = 7) to the systemic injection of cumulative doses of nicotine (**NIC**, 25–800 μ g/kg, *i.v.*) or saline (**SAL**, n = 7), following nicotine chronic treatment. Nicotine-induced response was no longer significantly different from the control group (Values represent mean \pm S.E.M.; 1-way ANOVA rm, followed by Fisher's PLSD post-hoc test; * $p < 0.05$, ** $p < 0.01$ vs VEH+SAL). Illustrative rate meters showing the lack of response to nicotine administration (b₁) and a control neuron (b₂).

FIGURE 3

Nicotine-induced responses in VTA DA neurons of drug-naïve rats.

Systemic administration of nicotine dose-dependently increased the neuronal activity of single VTA DA neurons in drug-naïve rats. Graphs show the mean (\pm S.E.M.) normalized change in basal FR induced by the systemic administration of nicotine as cumulative doses (25 - 800 $\mu\text{g}/\text{kg}$ *i.v.*; $n = 12$) when all neurons were pooled together (**A**) or split into latVTA ($n = 6$) (**B**) and mVTA ($n = 6$) (**C**) DA neurons. Examples of rate meters corresponding to the data summarized in the graphs are shown in, respectively, **a2/3**, **b1** and **c1**. Biphasic response by the high concentration of nicotine were occasionally recorded (i.e., **a3**). Data were analyzed using a 1-way ANOVA rm, followed by Fisher's PLSD post-hoc test; * $p < 0.05$, ** $p < 0.01$ vs Saline.

FIGURE 4

Nicotine-induced responses in VTA DA neurons of chronic nicotine rats.

Systemic administration of nicotine dose-dependently increased the neuronal activity of single VTA DA neurons in rats chronically treated with nicotine. Graphs show the mean (\pm S.E.M.) normalized change in basal FR induced by the systemic administration of nicotine as cumulative doses (25 - 800 $\mu\text{g}/\text{kg}$ *i.v.*; $n = 16$) when all neurons were pooled together (**A**) or split into latVTA ($n = 6$) (**B**) and mVTA ($n = 10$) (**C**) DA neurons. Examples of rate meters corresponding to the data showed in the graphs are shown in, respectively, **a2,3**, **b1** and **c1**. Data were analyzed using a 1-way ANOVA rm, followed by Fisher's PLSD post-hoc test; * $p < 0.05$, ** $p < 0.01$ vs Saline.

FIGURE 5

Nicotine-induced responses in VTA DA neurons of drug-naïve rats following the LHb lesion

Nicotine-induced responses of VTA DA neurons are strongly modified by the selective LHb lesion in drug-naïve rats in both the latVTA (**A**) and mVTA (**B**) nuclei of the VTA. Rate meters illustrating the activity of single DA neurons recorded from either sham-lesioned or lesioned rats are displayed in, respectively, **a1/b1** and **a2/b2** for both nuclei. (**a3**, **b3**) Dose-response graphs show the mean (\pm S.E.M.) normalized change in basal firing rate induced by the systemic administration of nicotine (25 - 800 $\mu\text{g}/\text{kg}$ *i.v.*) of DA neurons recorded from either sham-lesioned ($n = 15$) or lesioned rats ($n = 15$) localized in the latVTA and mVTA nuclei, respectively. Data were analyzed using a 1-way ANOVA rm, followed by Fisher's PLSD post-hoc test; * $p < 0.05$, ** $p < 0.01$ vs Sham-Lesioned group. two previous groups of non-lesioned rats, that received either nicotine ($n = 12$) or saline (control, $n = 8$; Fig. 3a).

FIGURE 6

Schematic drawings illustrating the circuits that are hypothesized to be driven by nicotine-induced activation of the lateral habenula (LHb) and the ventral tegmental area (VTA). The glutamatergic output of the LHb is pictured in green while the GABAergic efferents from the rostromedial tegmental nucleus (RMTg) to the VTA are in red. **(A)** In nicotine-naïve animals, systemic acute nicotine activates the LHb output (LHb red arrow with **A** = acute nicotine) inducing a direct activation of medially located VTA (mVTA) neurons and indirect inhibition of lateral VTA (latVTA) neurons mediated by RMTg (VTA red arrows with **A** = acute nicotine); following chronic nicotine, the LHb output is strongly reduced in response to a further challenge with nicotine (LHb red arrow with **C** = chronic nicotine), thus facilitating the inversion of the response pattern within the VTA. In chronic nicotine-treated rats a challenge of nicotine will now produce excitation of latVAT neurons while mVTA neurons do not respond with firing change **(B)** Following the LHb lesion, acute nicotine induced the same pattern of responses in the VTA observed for the chronic nicotine because like chronic nicotine the LHb lesion silences the LHb activity. In nicotine-naïve rats with the LHb lesioned, a challenge of nicotine will now produce excitation of latVAT neurons while mVTA neurons do not respond with firing change. These data suggest that the LHb might play a role in mediating nicotine-induced responses in the VTA at the level of different DA subpopulations that have been suggested to mediate reward and aversion.

