


Nicotine smoking concentrations modulate GABAergic synaptic transmission in murine medial prefrontal cortex by activation of $\alpha 7^*$ and $\beta 2^*$ nicotinic receptors

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Abstract

Nicotine is the major addictive component of cigarettes, reaching a brain concentration of ~300 nM during smoking of a single cigarette. The prefrontal cortex (PFC) mechanisms underlying temporary changes of working memory during smoking are incompletely understood. Here, we investigated whether 300 nM nicotine modulates γ -aminobutyric acid (GABA) ergic synaptic transmission from pyramidal neurons of the output layer (V) of the murine medial PFC. We used patch clamp in vitro recording from C57BL/6 mice in the whole-cell configuration to investigate the effect of nicotine on pharmacologically isolated GABAergic postsynaptic currents (IPSCs) in the absence or presence of methyllycaconitine (MLA) or dihydro- β -erythroidine (DH β E), selective antagonists of $\alpha 7^*$ - and $\beta 2^*$ -containing ($\alpha 7^*$ and $\beta 2^*$) nicotinic acetylcholine receptors (AChRs), respectively. Our results indicated that nicotine, alone or in the presence of MLA, decreases electrically evoked IPSC (eIPSC) amplitude, whereas in the presence of DH β E, nicotine elicited either an eIPSCs amplitude increase or a decrease. In the presence of DH β E, nicotine increased membrane conductance leaving the paired pulse ratio unchanged in all conditions, suggesting a non- $\beta 2^*$ mediated effect. In the presence of MLA, nicotine decreased the mean spontaneous IPSC (sIPSC) frequency but increased their rise time, suggesting a non- $\alpha 7^*$ AChR-mediated synaptic modulation. Also, in the presence of DH β E, nicotine decreased both eIPSC rise and decay times. No receptors other than $\alpha 7^*$ and $\beta 2^*$ appear to be involved in the nicotine effect. Our results indicate that nicotine smoking concentrations modulate GABAergic synaptic currents through mixed pre- and post-synaptic mechanisms by activation of $\alpha 7^*$ and $\beta 2^*$ AChRs.

KEYWORDS

central nervous system, gamma-aminobutyric acid, pair pulse, patch clamp

Abbreviations: AMPA, α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; DH β E, dihydro- β -erythroidine; DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; eIPSC, evoked inhibitory postsynaptic currents; GABA, gamma-aminobutyric acid; mPFC, medial prefrontal cortex; NMDA, N-methyl-D-aspartate; PFC, prefrontal cortex; PPR, paired pulsed ratio; sIPSC, spontaneous inhibitory postsynaptic currents; MLA, methyllycaconitine.

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1 | INTRODUCTION

(–)-Nicotine is the major addictive component of cigarettes, reaching brain concentrations of 300–500 nM during smoking (Matta et al., 2007). The alkaloid belongs to a most addictive class of drugs—second only to opioids—and yields a direct and indirect cost for human health in the order of 300 billion dollars per year in the US alone (Makate et al., 2019). Understanding nicotine neuronal mechanisms of action is of paramount importance in the fight against this pernicious addiction.

In the brain, (–)-nicotine activates different nicotinic acetylcholine receptor (nAChR) subtypes with distinct potency and efficacy. The mouse PFC has a particularly dense cholinergic innervation, where $\alpha 7^*$ and $\alpha 4\beta 2^*$ subtypes are mainly expressed (Poorthuis & Mansvelder, 2013). Nicotinic AChRs have been implicated in various neurological disorders, including drug (and nicotine) addiction, autosomal dominant frontal lobe epilepsy, schizophrenia, Tourette's syndrome, attention deficit hyperactivity disorder (ADHD), autism, depression, and Alzheimer's and Parkinson's diseases (Gotti, Zoli, & Clementi, 2006). The percentage of smokers among patients with neurological disorders, including dementia, ADHD, depression, and schizophrenia, is significantly higher than that in the normal population, suggesting that patients are seeking relief by nicotine self-medication (Bloem, Poorthuis, & Mansvelder, 2014).

Modulatory effects of nAChRs on γ -aminobutyric acid (GABA)ergic synaptic transmission had been previously reported in numerous brain areas, including the midbrain periaqueductal gray (Nakamura & Jang, 2010), hippocampus (Arnaiz-Cot et al., 2008; Banerjee, Alkondon, Pereira, & Albuquerque, 2012; Giocomo & Hasselmo, 2005; Lagostena et al., 2010; Wanaverbecq, Semyanov, Pavlov, Walker, & Kullmann, 2007), substantia nigra pars compacta (Xiao et al., 2009), dorsal subcoeruleus nucleus (Heister, Hayar, & Garcia-Rill, 2009), visual cortex (Lucas-Meunier et al., 2009), area postrema (Kawa, 2007), lateral hypothalamus (Jo & Role, 2002), frontal area 2 (Aracri, Meneghini, Coatti, Amadeo, & Becchetti, 2017), and mPFC (Aracri et al., 2010; Couey et al., 2007). This variety of actions makes utterly complex the behavioral significance of cellular and synaptic effects of nicotine in the mammalian neocortex and brain in general.

A crucial brain area for nicotinic modulation appears to be the neocortex. An important branch of cholinergic (and nicotinic) innervation projects from the basal forebrain to the whole neocortex (Semba, 2004). Among nAChRs potently activated by nicotine, the heteromeric subtypes $\alpha 4\beta 2$ - and $\alpha 6\beta 2$ -containing nAChRs ($\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$) (Gotti & Clementi, 2004; Poorthuis & Mansvelder, 2013). $\alpha 7$ -containing nAChRs ($\alpha 7^*$) appear to have lower affinities for acetylcholine and nicotine and display a faster

desensitization rate compared with $\beta 2^*$ nAChRs. Several nAChR subtypes (i.e. $\alpha 4\beta 2$, $\alpha 5\beta 2$, and $\alpha 7$) are expressed in the neocortex (Gotti et al., 2006), particularly in the medial PFC cortex (mPFC) (Poorthuis & Mansvelder, 2013), supplying a rationale why cholinergic stimulation to the mPFC improves attention performance, and represents one of the few relatively effective treatments of cognitive deficits in Alzheimer's disease and schizophrenia (Poorthuis & Mansvelder, 2013). The mPFC is a critical brain structure in volition and decision-making. Alteration of its inhibitory circuitry is deemed crucial for drug addiction and impulse control (Paine, Cooke, & Lowes, 2015; Poorthuis, Bloem, Verhoog, & Mansvelder, 2013).

While previous studies corroborated the existence of functional $\beta 2^*$ nAChRs in the PFC, several questions remain open about the nature of nicotinic modulation in PFC layer V, particularly whether a concentration of nicotine like the one reached after cigarette smoking may still produce a similar—or different—modulation of GABAergic synaptic currents. Our study aimed to determine whether and how smoking concentrations of nicotine modulates GABAergic synaptic currents in principal neurons of infragranular layer V. We monitored spontaneous inhibitory postsynaptic currents (sIPSC), as to compare our results with those obtained previously (Aracri et al., 2010, 2017), but we also studied nicotinic modulation of IPSCs electrically evoked by local electrical stimulation, in order to limit the source variability of the GABAergic fibers inherent with spontaneous synaptic release. We found that smoking concentrations of nicotine produce a complex effect by activating both $\alpha 7^*$ and $\beta 2^*$ nAChRs on murine PFC, whose overall result is to reduce inhibitory control.

2 | MATERIALS AND METHODS

2.1 | Brain slices

For this study, we used 50 mice (C57BL/6J) from both sexes (24 male, 26 female), aged between postnatal day 45 and 90, obtained from the animal facilities (Universidad Autonoma de San Luis Potosi). Animals were anesthetized in a closed container saturated with isoflurane (Baxter), killed following the Norma Nacional Mexicana (UASLP protocol number 2240, bioethics committee of the Faculty of Chemical Sciences) by decapitation, and their brains sliced with a vibrotome (VT1200, Leica) in a cold solution (0–4°C) containing (mM): 126 NaCl, 3.5 KCl, 10 glucose, 25 NaHCO₃, 1.25 NaH₂PO₄, 1.5 CaCl₂, 1.5 MgCl₂ at pH 7.4 (artificial cerebrospinal fluid, ACSF), and bubbled constantly with O₂/CO₂ (95%/5%) at pH 7.35 and osmolarity 300 mOsm. After removal of the olfactory lobes, coronal slices (270 μ m thick) were cut from the prefrontal cortex

and incubated in ACSF at 32°C before transferring them to the recording chamber.

2.2 | Electrophysiological recordings from the medial prefrontal cortex

To investigate the effects of nAChRs on GABAergic synaptic transmission at concentrations resembling human brain levels during smoking, 300 nM nicotine was bath-applied (~2 ml/min) onto mPFC brain slices at room temperature (22–23°C).

2.3 | Electrically evoked and spontaneous inhibitory postsynaptic currents

Synaptic responses were recorded in the mPFC, specifically in layer V pyramidal neurons (as shown in Figure 2c). Electrically evoked (eIPSCs) and spontaneous (sIPSCs) inhibitory postsynaptic currents were recorded with a glass electrode (4–5 MΩ) using patch clamp recording in the whole-cell configuration at a holding membrane potential $V_h = -60$ mV. Pyramidal cells were visually identified with an up-right microscope (BX-51 Olympus) and an infrared camera system (DAGE-MT). Evoked IPSCs (eIPSC) were induced by electrically stimulating cortical layer II–III (as shown in Figure 2c), using an isolated stimulation unit and a glass electrode (4–5 MΩ resistance, filled with ACSF). Glass electrodes were filled with an intracellular solution containing (in mM): 100 CsCl, 5 1,2-bis (2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid K (BAPTA-K), 1 lidocaine N-ethyl bromide (QX314), 1 MgCl₂, 10 N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid), 4 glutathione, 3 ATPMg₂, 0.3 GTPNa₂, 20 phosphocreatine, and 7 biocytin. The holding voltage was not corrected for the junction potential (<4 mV). The intracellular recording solution was previously titrated to pH 7.35 and osmolarity 270 mOsm. Electrically evoked inhibitory postsynaptic currents (eIPSC) were measured by delivering two identical electric stimuli (90–180 μs, 70–100 μA) 200 ms apart (paired stimulation) every 12 s. Synaptic responses were monitored at different stimulation intensities, and the intensity evoking ~60% of the maximal response was used to elicit evoked responses from each recording. Basal eIPSCs GABAergic currents displayed an amplitude range from 97 to 1,389 pA (mean ± SEM = 359.4 ± 41.7), at $V_h = -60$ mV. Evoked IPSCs were recorded during 10–15 min until a stable basal response was obtained, before drug application.

2.4 | Drugs and solutions

Stock solutions of each drug, including (–)-nicotine, 6,7-dinitroquinoxaline-2,3-dione (DNQX), kynurenic acid, MLA, and DHβE, were prepared in distilled water and diluted in ACSF to reach the final concentration to be applied

onto mPFC brain slices. Most of the used drugs were purchased from Sigma (Sigma-Aldrich Corp.), whereas DHβE was obtained from Tocris (Tocris Bioscience), and MLA from Abcam (Abcam biochemical). Avidin–biotin complex (1:500, Vector Elite), gelatin (Merck KGaA), Entellan® embedding agent (Merck KGaA). In order to pharmacologically isolate inhibitory postsynaptic GABAergic currents (IPSCs), we performed all experiments in the presence of the glutamate receptor (GluR) blockers 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX 10 μM, blocker of the amino-propionic acid (AMPA) type GluRs, and either kynurenic acid (2 mM) or amino phosphovaleric acid (AP5, 100 μM) for blocking N-methyl-D-aspartate (NMDA) type GluRs. As nicotine produced similar effects in the presence of kynurenic acid or AP5, the results from these experiments were pooled together. To pharmacologically differentiate the role of each nAChR subtype on nicotine activity, the α7*- and β2*-selective antagonists methyllycaconitine (MLA, 30 nM) and dihydro-β-erythroidine (DHβE, 400 nM) were applied alone or together in combination with the beginning of each experiment as indicated in the text.

2.5 | Biocytin staining

Brain slices were immediately fixed by immersion in phosphate buffer (0.01 M, pH 7.2) 4% paraformaldehyde for 48 hr and then transferred to 30% sucrose-0.04% NaN₃ in Phosphate Buffer Saline (0.01 M, pH 7.6). After rinsing, sections were incubated in avidin–biotin complex (1:500) in diluted 0.01 M Tris Buffered Saline, containing 0.5% Triton X-100 and 0.25% for 2 hr. The final reaction was performed with a solution of 3,3'-diaminobenzidine 0.25% and H₂O₂ 0.01% diluted in Tris Buffered Saline for 10 min. Slices were mounted on gelatinized slides, dried, rinsed in distilled water and then stained for Nissl substance (Cresyl Violet acetate 0.1% and 0.3 ml of acetic acid in distilled water) at 37°C for 5 min. After a quick rinse in distilled water, sections were dehydrated with graded solutions of ethanol (70%, 96% and 100%) and xylene and cover slipped with Entellan embedding agent. Digital pictures of the cells were taken by using an Axio ScopeA.1 microscope equipped with a digital color photcamera ERc5s (both provided by Zeiss).

2.6 | Data analysis

Results were expressed as mean ± SEM. The number of recordings in each experiment is indicated as “n”. At most, three recordings were performed in the same animal, and a minimum of three animals were used per each experiment. Paired Student's *t* test was used to evaluate the differences between mean values obtained from the same group of cells under two different experimental conditions. Unpaired Student's *t* test was used to assess the data obtained from

FIGURE 1 Effect of the application of nicotine (300 nM) on eIPSCs amplitude from PFC layer V pyramidal neurons. (a) Temporal course of the amplitude of the first response induced by paired pulse stimulation of layer II/III (see Methods) before and following bath application of nicotine in control conditions (no nAChR antagonist present). Line on top of the time course indicates the time of bath application of 300 nM nicotine. Evoked IPSC amplitude changed from 384 ± 55 pA in control to 257 ± 39 pA in the presence of nicotine (paired *t*-Student test, $p < 0.01$, $n = 12$). In the insert, each trace shows the average of four traces before (black) or after (red) nicotine application. (b) Scatter plots illustrate individual eIPSC amplitude change before and after nicotine application, red indicates statistically significant increases; blue: decreases; black: no change. (c and d) same legend as a and b, in the presence of the $\alpha 7^*$ -selective antagonist MLA (30 nM). The average eIPSC amplitude in the presence of 30 nM MLA alone was 327 ± 36 pA, which decreased to 146 ± 24 pA in the presence of 300 nM nicotine plus MLA (paired *t*-Student test, $n = 9$, $p < 0.01$). (e and f) same legend as above, but in the presence of the potent $\alpha 4\beta 2^*$ nAChR antagonist DH β E (400 μ M) for the cell population responding to nicotine with an increment. Average eIPSCs amplitude increased from 301 ± 41 pA to 409 ± 59 pA in the presence of 300 nM nicotine (paired *t* test, $n = 10$ out of 19, $p < 0.01$). (g and h) In the presence of the same concentration of DH β E but for the cell population responding to nicotine with an eIPSC amplitude decrease from 630 ± 93 pA to 506 ± 75 pA in 8/19 recordings (paired *t* test, $p < 0.01$). (i and j) represent the effect of nicotine in the presence of both antagonists (MLA plus DH β E, same concentrations as above). All recordings were performed in the presence of kynurenic acid (2 mM) or 100 μ M AP5 and DNQX (10 μ M). The eIPSC amplitude value (375 ± 45 pA) did not change after application of 300 nM nicotine (333 ± 42 pA), paired *t*-Student test, $n = 10$, $p < 0.10$)

different cell groups. Unpaired *t* test was also used to assess differences between statistically stable periods in control and treatment within single recordings. A statistically stable period was defined as a time interval (5–8 min) along which the mean eIPSC amplitude measured during any couple of assessments each corresponding to 1 min did not vary from each other according to an unpaired Student's *t* test. Outliers (mean $> 2\sigma$) were included in the mean calculation but were removed from figures for clarity. Two-tailed $p < 0.05$ values were considered statistically significant. Only statistically significant results were reported unless specified differently.

The paired pulse ratio (PPR) was calculated as the mean amplitude response of the second peak response electrically induced divided by the mean first response, as in Kim & Alger (2001). We used the Clampfit software (pClamp, Molecular Devices, Axon) to analyze IPSCs. MiniAnalysis program to analyze sIPSCs and Sigma plot to analyze all data.

3 | RESULTS

3.1 | Nicotine decreases eIPSCs amplitude

The effect of 300 nM nicotine was investigated by adding it to the perfusion solution during 10–15 min after a stable baseline was reached. Nicotine decreased the amplitude of GABAergic currents in 10/12 recordings, reaching its maximal effect ~10 min after starting its perfusion, in 1/12 recordings it increased and in 1/12 did not change eIPSC amplitude. As the overall effect was inhibitory, the mean effect of nicotine was calculated over the whole sample ($n = 12$). Representative traces and time course of the nicotine effect are shown respectively in the insert at top and bottom of Figure 1a. Figure 1b displays the effect of nicotine on eIPSC amplitude of individual recordings. The average eIPSC amplitude in control was 384 ± 55 pA, vs. 257 ± 39

pA in the presence of nicotine (paired *t*-Student test, $n = 12$; $p = .0009$). PPR value did not significantly change in the presence of nicotine.

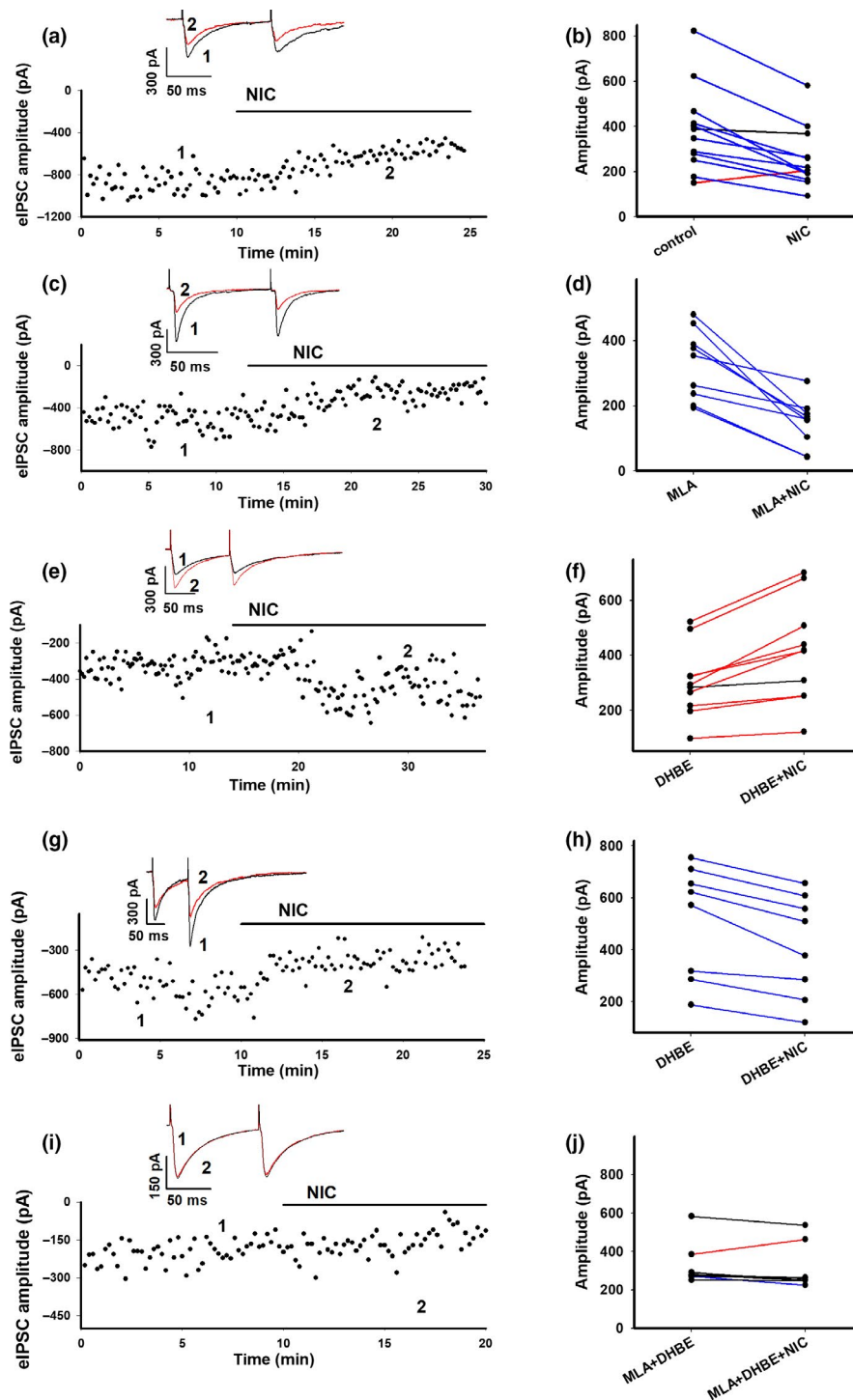
As the most common nAChR subtypes present in murine cortex are $\alpha 7^*$ and $\alpha 4\beta 2^*$ nAChRs (Gotti et al., 2006), their role in the inhibitory effect of nicotine on eIPSCs was subsequently investigated by using MLA and DHBE, which selectively inhibit $\alpha 7^*$ and $\beta 2^*$ nAChRs, respectively (Alkondon, Pereira, Eisenberg, & Albuquerque, 1999; Maggi, Sher, & Cherubini, 2001).

3.2 | $\beta 2^*$ nAChRs mediate the inhibitory effect elicited by nicotine on eIPSCs

Application of nicotine in the presence of MLA decreased eIPSC amplitude in 9/9 recordings. Figure 1c–d show a representative time course and amplitude scatter plots, respectively (representative recording in insert of Figure 1c). The average amplitude value of eIPSC in the presence of 30 nM MLA alone was 327 ± 36 pA, which decreased to 146 ± 24 pA in the presence of 300 nM nicotine (plus 30 nM MLA, paired Student *t* test, $n = 9$ cells, $p = .0006$, Figure 1d). PPR value in MLA did not change in the presence of nicotine.

3.3 | Bimodal modulation of eIPSCs amplitude by $\alpha 7^*$ nAChRs

Application of nicotine in the presence of DH β E increased eIPSCs amplitude in 10/19 pyramidal cells tested (representative time course in Figure 1e, individual amplitudes in Figure 1f), whereas it decreased eIPSC amplitude in 8/19 recordings (representative time course in Figure 1g, individual amplitudes in Figure 1h). In the former, the average value of eIPSCs amplitude in the presence of 400 nM DH β E was increased from 301 ± 41 pA (alone) to 409 ± 59 pA in the presence of 300 nM nicotine (paired *t* test, $n = 10$ cells,



$p = 0.001$, Figure 1f). In the latter population, under the same conditions, nicotine decreased eIPSCs amplitude from 630 ± 93 pA to 506 ± 75 pA in 8/19 recordings (paired t test, $p = 0.002$, individual amplitudes in Figure 1h), whereas nicotine yielded no effect in 1 out of 19 cells (in Figure 1f, black). The average PPR value for DH β E did not change in the presence of nicotine in either group. In 3 of the 5 animals in which nicotine was tested on more than one recording (in the presence of DH β E), nicotine-induced eIPSC amplitude increase in at least one cell and decrease in at least another

cell, suggesting that the $\alpha 7$ -dependent modulation is not necessarily homogeneous within a single animal.

3.4 | Only $\alpha 7^*$ and $\beta 2^*$ nAChRs are responsible for the eIPSCs modulation

Simultaneous application of both selective antagonists (MLA and DH β E) prevented nicotine modulation of eIPSCs in 8/10 recordings, while it yielded an amplitude increase and an amplitude decrease in 1 cell each. Figure 1i shows

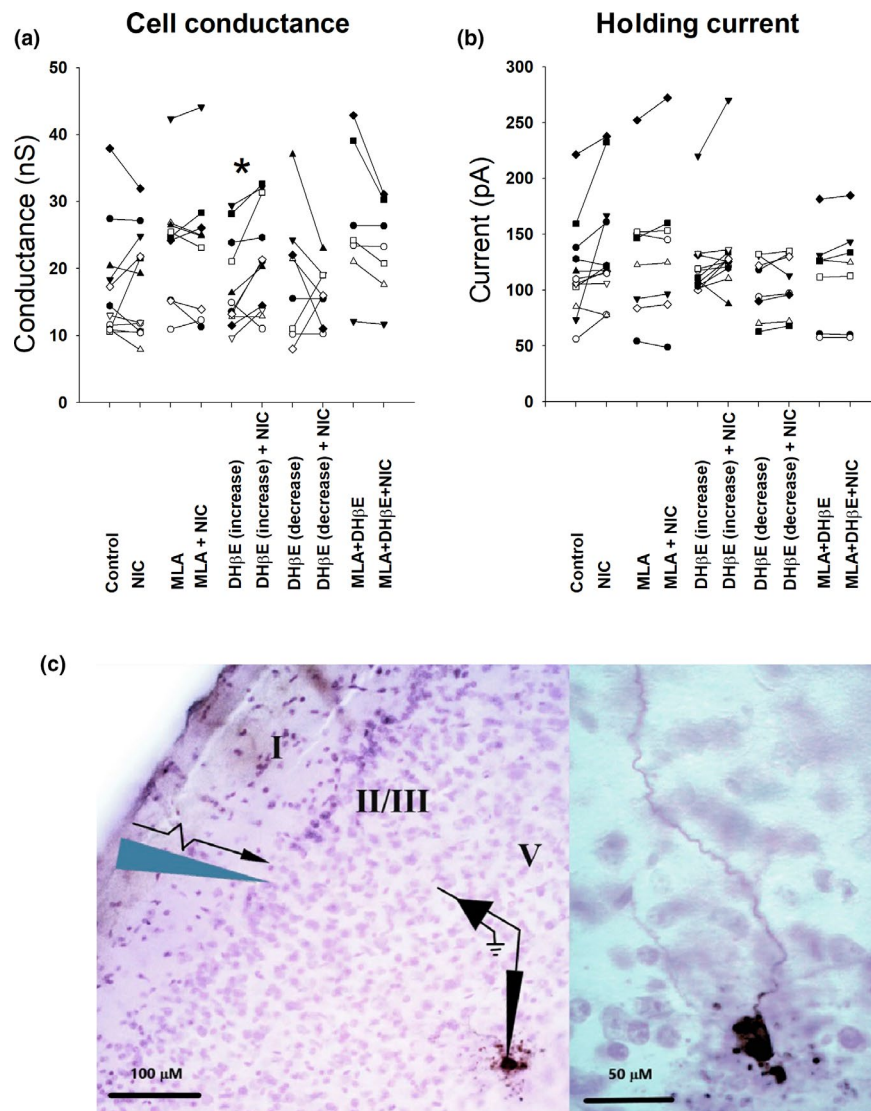


FIGURE 2 $\alpha 7^*$ nAChRs activation increases the conductance of pyramidal neurons. The conductance of the recorded neurons was monitored using whole cell, patch clamp and voltage clamp, at $V_h = -60$ mV, by applying a -5 mV pulse during the experiments and recording the current in this condition in each sweep. (a) Scatter plot shows the conductance measured with a -5 mV pulse before and after the application of nicotine (300 nM alone [control ($n = 12$)], MLA ($n = 9$), DH β E (increase; $n = 10$), DH β E (decrease; $n = 8$), and MLA + DH β E ($n = 7$)). An increase of the conductance was observed only in the DH β E increase group (3.6 ± 0.4 pS before nicotine application vs. 4.4 ± 0.6 pS after nicotine application, paired t -Student test, $n = 8$ cells, $p = 0.043$). (b) Scatter plot shows the holding current of each drug, in the absence and the presence of nicotine (same sample as panel A). Nicotine (300 nM) did not modify the holding current in any of the tested conditions. Asterisk indicates statistically significant differences ($p < 0.05$) between groups by using the paired Student's t test. (c) Photomicrograph of a representative recorded pyramidal neuron from layer V mPFC filled with biocytin and staining Nissl substance, electric stimulation was placed in layer II-III and the recordings in layer V, left and right scale bars 100 and 50 μ m, respectively

representative traces and amplitude time course (above and below, respectively) from a layer V pyramidal neuron in the presence of both $\alpha 7^*$ - and $\beta 2^*$ -selective blockers, respectively. Mean eIPSC amplitude value was 375 ± 45 pA in the presence of 30 nM MLA plus 400 nM DH β E versus 333 ± 42 pA after simultaneous application of 300 nM nicotine (plus 30 nM MLA and 400 nM DH β E, paired t test, $n = 10$ cells, $p = 0.12$, individual amplitudes in Figure 1j). In addition, the

average PPR value for MLA + DH β E did not change in the presence of nicotine.

3.5 | $\alpha 7^*$ nAChR activation increases the conductance of pyramidal cortical neurons

To gather information about possible postsynaptic effects of nicotine, we investigated whether nicotine

induces any change in the conductance of the recorded pyramidal neurons. Membrane conductance was monitored during the recordings by applying a -5 mV pulse at the beginning of each sweep and before eIPSCs and recording the current induced. Figure 2a summarizes the average membrane conductance values in basal conditions for each experiment (control, MLA, DH β E increase and decrease, and MLA + DH β E) and in the same solutions after nicotine (300 nM) bath application. Nicotine did not change neuronal conductance either in the presence of MLA alone, or in MLA plus DH β E. However, nicotine increased neuronal conductance in the presence of DH β E in the group in which nicotine increased eIPSC amplitude (3.6 ± 0.4 pS before nicotine application to 4.4 ± 0.6 pS after nicotine application, paired t test, $n = 8$ cells, $p = 0.043$). These results suggest postsynaptic changes induced by $\alpha 7^*$ nAChRs. Additionally, changes in the holding current in control (control, MLA, DH β E, and MLA + DH β E) conditions and in the presence of nicotine (300 nM) were also investigated. The average holding current values in any condition were not different to those in the presence of nicotine (Figure 2b). A representative biocytin stain from a recorded pyramidal cell is shown in Figure 2c.

3.6 | Nicotine decreases the frequency of sIPSCs in cortical pyramidal neurons

We also investigated whether smoking nicotine concentrations modulate spontaneous IPSCs (sIPSCs, Figure 3a–l). Nicotine decreased the mean frequency of sIPSCs from 2.1 ± 0.3 Hz (before) to 1.4 ± 0.3 Hz (after nicotine bath application, paired t test, $n = 7$ cells, $p = 0.047$) but not the mean sIPSC amplitude. Individual variation of sIPSC frequency and amplitude are shown in Figure 3b above and below, respectively. Examples of cumulative probability curve of interevent interval and amplitude are displayed in Figure 3c (above and below, respectively). Notice a significant nicotine-induced right shift of the eIPSC frequency, with no change in the average sIPSC peak amplitude.

The observed decrease in mean frequency of sIPSCs was still apparent in the presence of MLA (representative traces in Figure 3d), with average values of 1.3 ± 0.3 Hz (before) and 0.8 ± 0.2 Hz (after bath application of nicotine, paired t test, $n = 9$ cells, $p = 0.038$), as indicated by the individual changes in Figure 3e (above) and by the right shift in cumulative probability curve of interevent interval (Figure 3f above). No change was found in the average peak amplitude (Figure 3e, below) nor in cumulative probability amplitude (Figure 3f, below). Nicotine did not modify the mean frequency or mean peak amplitude of sIPSCs in the presence of DH β E (Figure 3g–i, same legend as above), nor in the presence of MLA plus DH β E (representative recordings in Figure

3j). Amplitude and cumulative probability plots of interevent interval and peak amplitude are displayed in Figure 3k,l, respectively (same legend as for the previous panels). These results suggest the presence of presynaptic $\beta 2^*$ nAChRs in GABAergic interneurons.

3.7 | Nicotine smoking concentrations modify kinetic parameters of sIPSCs by $\alpha 7^*$ or $\beta 2^*$ nAChR activation

Nicotine changed neither the rise nor decay time constants (calculated between 20% and 80%) in the absence of any nAChR inhibitor. However, nicotine increased the rise time of sIPSCs from 3.0 ± 0.1 ms to 3.5 ± 0.2 ms in the presence of MLA (paired t test, $n = 9$ cells, $p = 0.02$), whereas an opposite effect was observed in the presence of DH β E, where the rise time decreased from 5.3 ± 0.4 ms to 3.8 ± 0.3 ms and the decay time from 16.4 ± 0.7 ms to 14.4 ± 0.4 ms ($n = 7$ cells, $p = 0.01$ for both). In the presence of both MLA and DH β E, nicotine did not modify the kinetic parameters of sIPSC (Figure 4a–c). Altogether, these data suggest a postsynaptic effect of GABAergic transmission on pyramidal neurons induced by both $\alpha 7^*$ and $\beta 2^*$ nAChRs.

3.8 | Nicotine does not modify muscimol-induced currents

We also investigated whether smoking nicotine concentrations modify the currents induced by the GABA $_A$ R agonist muscimol in layer V pyramidal neurons from mPFC. Muscimol was applied locally by puffs using a Picospritzer III (6 PSI, 10 ms). Currents induced by 100 μ M muscimol were recorded every 60s using whole-cell voltage clamp ($V_h = -60$ mV) before (basal) and during (15 min) bath application of 300 nM nicotine (representative time course and traces in Figure 5a,b, respectively). Traces in display are the mean of 4 individual traces before (black) and after (red) nicotine (300 nM) administration. Nicotine did not change the current induced by muscimol (paired t test, $p = .96$, $n = 7$), where the average currents induced by muscimol in extracellular cerebrospinal fluid was 621.2 ± 217.8 pA and 618.5 ± 214.5 pA in the absence and presence of 300 nM nicotine, respectively. Mean \pm SEM of the peak muscimol-induced current is shown in Figure 5c. Application of 20 μ M bicuculline, a GABA $_A$ R antagonist, completely inhibited the currents induced by muscimol, corroborating the GABAergic nature of the muscimol-induced currents (green).

4 | DISCUSSION

This is the first report showing that a nicotine smoking concentration decreases eIPSCs in mPFC pyramidal neurons.

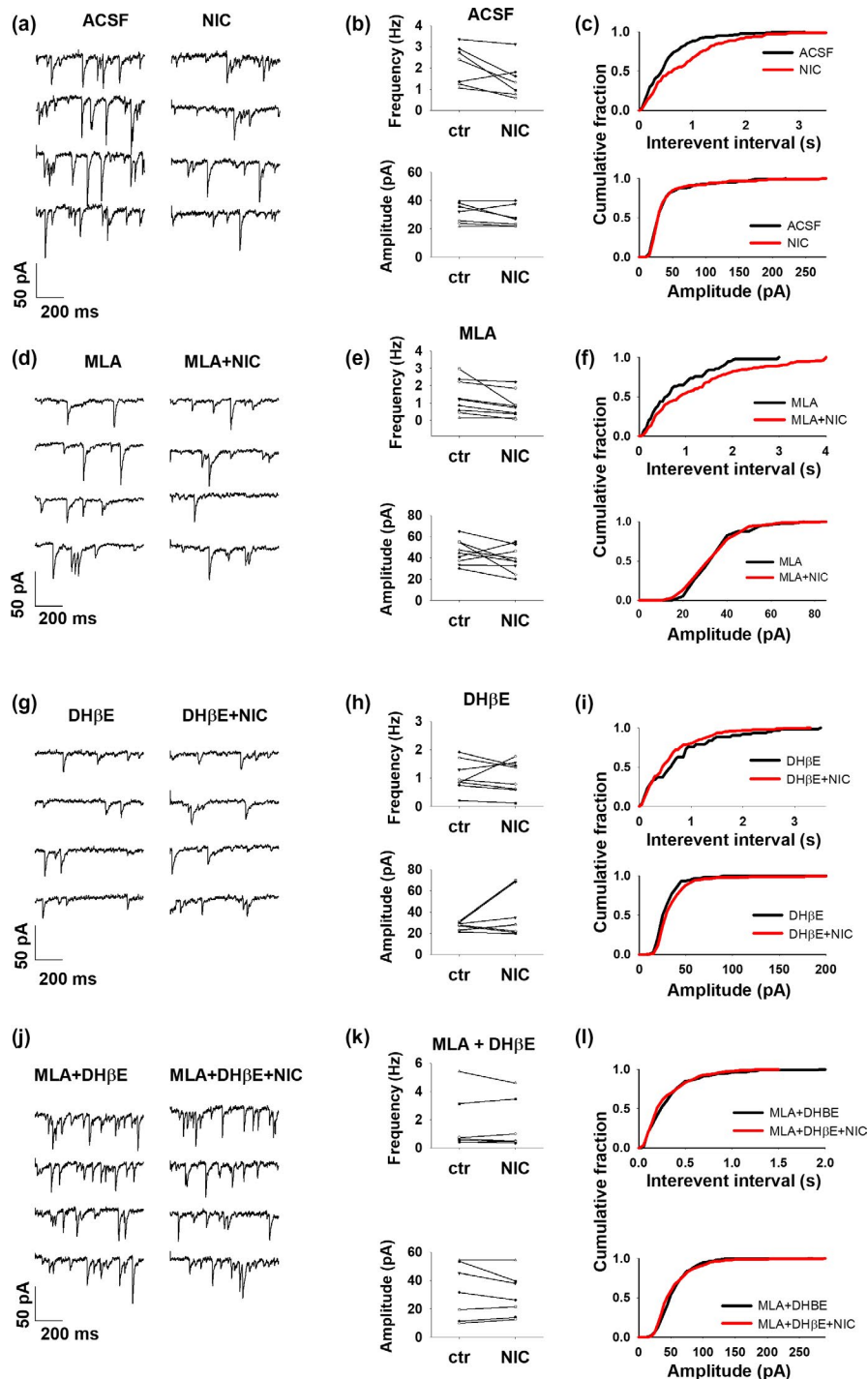


FIGURE 3 $\beta 2^*$ nAChR activation decreases the frequency of sIPSCs in layer V pyramidal neurons from mPFC. (a, d, g, and j) Representative recordings obtained from pyramidal neurons displaying spontaneous inhibitory postsynaptic currents (sIPSCs) in basal conditions (control, MLA, DH β E, or MLA + DH β E) and in the presence of 300 nM nicotine plus nicotinic inhibitors. Scatter plots display the frequency or amplitude (b, e, h, and k) of sIPSCs from pyramidal neurons. Nicotine decreases the frequency of sIPSC (2.1 ± 0.3 Hz before vs. 1.4 ± 0.3 Hz after nicotine bath application, paired *t*-Student test, $n = 7$ cells, $p = 0.047$). The effect was still present in the presence of MLA (30 nM, 1.3 ± 0.3 Hz before vs. 0.8 ± 0.2 Hz after bath application of nicotine, paired *t*-Student test, $n = 9$ cells, $p = 0.038$). Graphs represent the distribution of the interevent interval or peak amplitude (c, f, i and l) in basal conditions (control, control + MLA, control + DH β E, or control + MLA and DH β E) and in the presence of 300 nM nicotine, graphs were obtained from the corresponding cell on the left

Nicotine-induced eIPSC inhibition was not blocked by application of the $\alpha 7^*$ -selective inhibitor (MLA). In the presence of DH β E, however, nicotine applications elicited either an increase or a decrease in eIPSCs amplitude in about half of the recordings, respectively. The simultaneous application of both inhibitors completely blocked both effects elicited by nicotine, corroborating the assumption that only $\alpha 7^*$ and $\beta 2^*$ nAChRs are responsible for these effects.

Altogether, these data suggest that 1) only $\alpha 7^*$ and/or $\beta 2^*$ nAChRs are involved in nicotinic-induced modulation of eIPSC amplitude of mice mPFC, 2) $\beta 2^*$ (possibly $\alpha 4\beta 2^*$

and/or $\alpha 6\beta 2^*$) nAChRs activation consistently induces a reduction in eIPSC amplitude, and 3) activation of $\alpha 7^*$ nAChRs produces opposite effects (increase or decrease) in two different neuronal populations. This finding indicates slight differences between the PFC and other brain areas, like the hippocampus, where MLA completely blocks the decrement of eIPSCs amplitude induced by ACh, suggestive of the exclusive participation of $\alpha 7^*$ nAChRs in nicotine modulation (Wanaverbecq et al., 2007).

Activation of $\alpha 7^*$ nAChRs also induced a small but statistically significant increase in cell conductance, possibly

FIGURE 4 Nicotine modifies the kinetic parameters of sIPSCs in pyramidal neurons. (a) In the presence of MLA, nicotine (300 nM) increased the sIPSC rise time from 3.0 ± 0.1 ms to 3.5 ± 0.2 ms (paired *t*-Student test, $n = 9$ cells, $p = 0.02$), while in the presence of DH β E both the rise time and the decay times were decreased, the former from 5.3 ± 0.4 ms to 3.8 ± 0.3 ms and the latter from 16.4 ± 0.7 ms to 14.4 ± 0.4 ms ($n = 7$ cells, $p = 0.01$ for both, paired *t*-Student test, panel b). Asterisks indicate statistically significant differences ($p < 0.05$) between groups by using the paired Student's *t* test. (c) Representative sIPCs in the different conditions tested (control, control + MLA, control + DH β E, or control + MLA and DH β E; indicated by numbers 1–4, respectively) in the absence (in black) and presence (in red) of nicotine (300 nM)

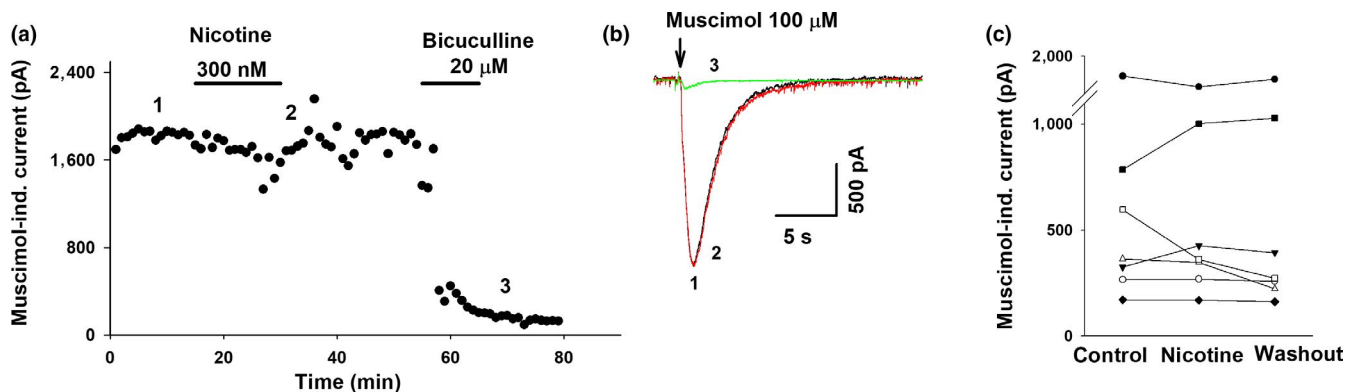
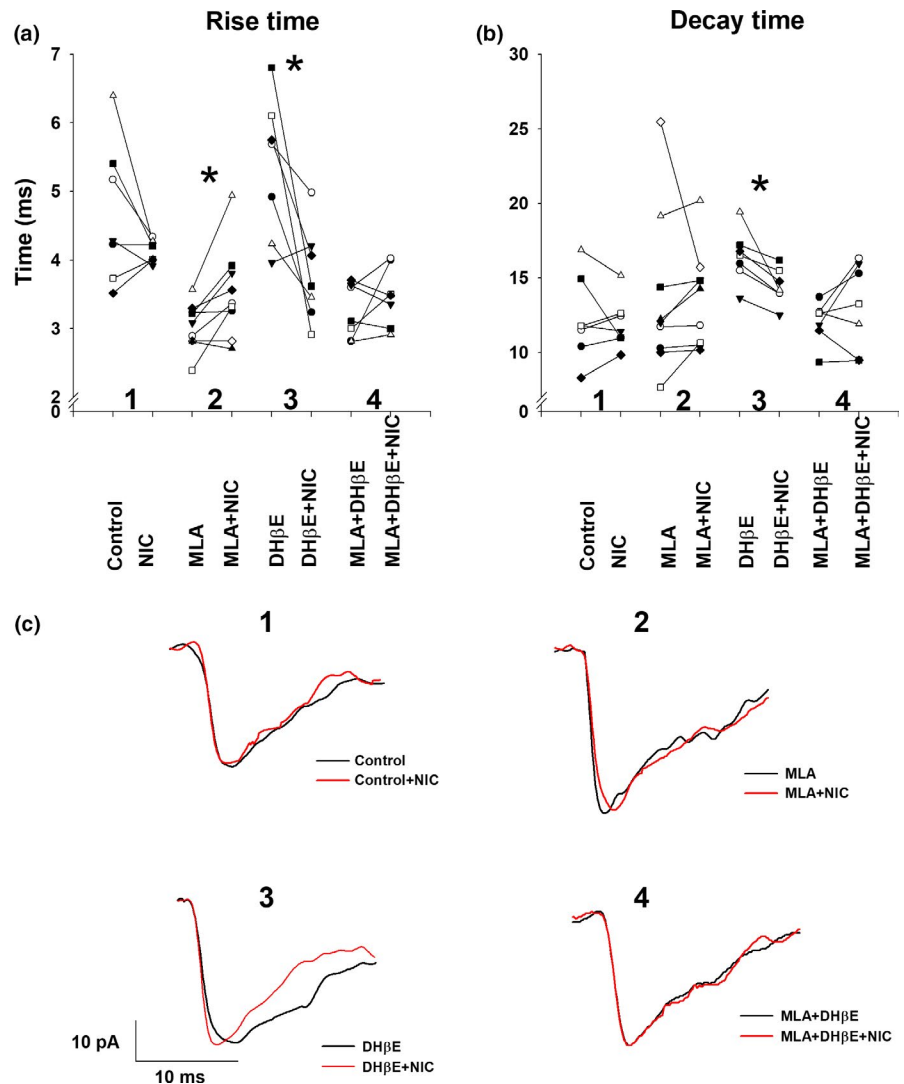


FIGURE 5 Nicotine did not change muscimol-evoked postsynaptic responses. (a) Representative time course of the effect of bath-applied nicotine (300 nM) on the chemically evoked IPSC. Muscimol was applied locally by puffs using a Picospritzer III (6 PSI, 10 ms). Currents induced by 100 μ M muscimol were recorded every 60 s using whole-cell voltage clamp ($V_h = -60$ mV). Nicotine and bicuculline applications are indicated by the bars. (b) Representative traces of the postsynaptic response evoked by application of muscimol (100 μ M). Each trace displayed is the average of four traces (black: before nicotine; red: after nicotine; and green: after bicuculline application). (c) Summary of the averages from the muscimol-evoked currents. No significant changes were detected after application of nicotine (618.5 ± 214.5 pA in control, vs. 621.2 ± 217.8 pA in the presence of 300 nM nicotine, $p > 0.10$, paired *t*-Student test, $n = 7$)

due to opening of Ca^{2+} -dependent K^{+} -channels. This data, together with a faster sIPSC kinetics following DH β E, suggest (at least) a postsynaptic location of $\alpha 7^{*}$ nAChRs. The failure of nicotine to modulate muscimol-induced currents on pyramidal neurons may be caused by preferential activation by muscimol of extra-synaptic (vs. intrasynaptic) GABA_A receptors, possessing a subunit composition (and modulation) differing from (intra) synaptic GABA_A receptors. This possibility is consistent with results obtained using ACh to stimulate the rat neocortex (Zolles et al., 2009). This and other studies have suggested the presence of nAChRs in pyramidal cells (Aracri et al., 2010; Zolles et al., 2009), as well as in GABAergic interneurons in the same brain area (Aracri et al., 2017).

Functional evidence of nicotinic modulation of neocortical GABAergic synaptic transmission had been described previously in the visual cortex (Lucas-Meunier et al., 2009), in frontal area 2 (Aracri et al., 2017) and in the mPFC (Aracri et al., 2010; Couey et al., 2007). In this last work, opposite results (IPSC frequency increase or decrease) were reported on the effects of 5 μM nicotine on GABAergic spontaneously evoked and miniature synaptic currents depending on the manner in which GABAergic currents were isolated: when isolated pharmacologically by using AP5 and cyanquinoxaline (CNQX) as blockers of glutamate ionotropic receptors, postsynaptic sIPSC frequency was decreased by nicotine, whereas sIPSC frequency was increased whenever measured at the reversal potential for AMPA currents, in two different sets of experiments. This puzzling result was explained by a different tone of glutamatergic input to local GABAergic interneurons. In the same study, nicotine enhanced the frequency of mIPSCs, effect that was blocked by DH β E, revealing a role of pre-synaptic $\beta 2^{*}$ nAChRs. Similarly, Couey et al., (2007) found that nicotine (10 μM) increased the frequency of sIPSCs in the absence of GluR antagonists, blocked by the unspecific antagonist mecamylamine but not by MLA, suggesting a role for non- $\alpha 7$ nAChRs.

In our study (IPSCs pharmacologically isolated), nicotine consistently decreased sIPSCs frequency, corroborating a role for $\beta 2^{*}$ nAChRs in GABA release. Discrepancies between ours and the previous results may be due to the fact that we used a 16-fold lower nicotine concentration. At this concentration (300 nM) $\alpha 4\beta 2^{*}$ nAChRs are presumably an order of magnitude more susceptible to be activated compared to $\alpha 7^{*}$ nAChRs. Our results are consistent with a post- and pre-synaptic location of $\alpha 7^{*}$ nAChRs (Poorthuis & Mansvelder, 2013), in agreement with the depolarization of GABAergic interneurons by nicotine local applications (Couey et al., 2007). Pre- and post-synaptic mechanisms of GABAergic synaptic transmission have been associated previously with differential expression of different nAChR subtypes in multiple brain areas (Gotti et al., 2006; Poorthuis

& Mansvelder, 2013), including hippocampus (Lagostena et al., 2010), dorsal subcoeruleus nucleus (Heister et al., 2009), and PFC (Flores-Hernandez et al., 2009; Tang et al., 2015; Verhoog et al., 2016).

In conclusion, our study suggests that nicotine bath applications at 300 nM activates nAChRs in their low activation range (reported EC_{50} values for $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in heterologous expression are 90 μM and 15 μM , respectively), while it is also possible that at this concentration a large proportion of the $\alpha 4^{*}$ and $\beta 2^{*}$ subunits nAChRs are at least partly desensitized (Fenster, Rains, Noerager, Quick, & Lester, 1997; Poorthuis et al., 2013). In the end, although nicotine from smoke appears to activate $\alpha 7^{*}$ and $\beta 2^{*}$ nAChRs in the low end of their activation range, its action is sufficient to alter in a potent and complex manner GABAergic transmission. Our data support the possibility that in juvenile or adults neurons, smoking concentrations of nicotine negatively modulate mPFC GABAergic interneurons—probably fast-spiking parvalbumin positive ones—by activating $\beta 2^{*}$ nAChRs, whereas the effect of the activation of $\alpha 7^{*}$ nAChRs on the inhibitory mPFC neurons by smoking nicotine is not homogeneous and has at least a postsynaptic origin. While our data contribute to shed some light on the synaptic effects of nicotine smoke on attention, working memory, and control inhibition, still more work needs to be done to further unravel nicotine-mediated smoke-induced physiologic mechanisms of action on mammalian brain and behavior.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

RCO conducted and analyzed most of the experimental data, EER obtained experimental data, RSD and NS obtained the images from fixed brain slices, MA designed and interpreted all the experiments, edited the manuscript, and supervised the project, HA gave the initial idea on the use of smoking nicotine concentrations, and HRA and CBL interpreted experimental data and edited the manuscript, MMM obtained, analyzed, and interpreted experimental data and wrote the manuscript. All authors contributed to the discussion of the results and the preparation of the manuscript.

DATA AVAILABILITY STATEMENT

All data presented in the current manuscript can be obtained from the corresponding author upon request.

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